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Investigation of vascular dysfunction in pre-clinical models of sepsis

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Investigation of vascular dysfunction in pre-clinical models of sepsis

Thesis submitted for the degree of

Doctor of Philosophy

King's College London

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| ABSTRACT

Sepsis is an overwhelming inflammatory response to infection that can progress to septic shock, characterised by refractory hypotension and insufficient organ perfusion. It is predominantly associated with nosocomial infections, and tends to occur in extremes of age, where it is associated with very high mortality rates. Sepsis and sepsis-associated multi-organ failure represent a tremendous healthcare burden and a significant cause of morbidity and mortality worldwide. Incidence has continued to increase over several decades, and septic shock is now the leading cause of death in intensive care units, claiming an estimated 20,000 lives per day worldwide.

Despite decades of endeavour, research has not produced any specific pharmacological treatments shown definitively to improve survival in septic patients. Cardiovascular collapse plays a key role in mortality, and correspondingly, pre-clinical and clinical assessment of the impact of interventions on disease progression has been based upon systemic haemodynamics and achieving target blood pressures. In recent years, however, it has become increasingly clear that systemic stabilisation does not necessarily prevent the onset of organ failure, and may actually be achieved at the expense of visceral perfusion. Sepsis is now increasingly understood to be a 'disease of the microcirculation', though pre-clinical models rarely assess this parameter, perhaps accounting for the lack of research translatability. There is thus a critical need to develop more clinically relevant pre-clinical models, which focus on clinically prognostic endpoints, in order to identify novel drug targets for the treatment of sepsis.

This thesis will describe the development of a novel approach to monitoring cardiovascular dysfunction in two widely used pre-clinical models of sepsis: lipopolysaccharide (LPS)-induced endotoxaemia, and cecal ligation and puncture (CLP). This approach comprises a multi-parameter monitoring system in which all levels of the cardiovascular system – global haemodynamics, cardiac function, microcirculatory blood flow and haematological markers – can be evaluated in a single animal. Using a novel application of laser speckle contrast imaging technology, in combination with gold standard haemodynamic monitoring techniques, we demonstrate that mesenteric blood flow is severely and time-dependently decreased following the induction of sepsis, despite stabilisation of arterial pressure, heart rate and cardiac output. Decreased mesenteric perfusion is shown to correlate with the development of metabolic acidosis and organ dysfunction, suggesting that global haemodynamic indices are insufficiently predictive of syndrome progression. Mesenteric blood flow, on the other hand, appears to be a sensitive and clinically relevant prognostic marker of disease severity, correlating with

haematological markers of organ dysfunction, and sensitive to standard clinical interventions known to improve survival in patients.

This thesis will go on to describe a mechanistic investigation of sepsis-induced microcirculatory flow impairment, using vasodilatory and anti-coagulant agents. Finally, it will describe the use of this model, in addition to *in vitro* models, for assessing the potential role of two ion channels – transient receptor potential vanilloid (TRPV) 1 and 4 – in modulating vascular dysfunction during sepsis.

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| ABBREVIATIONS

$[Ca^{2+}]_i$	intracellular calcium concentration
4 α PDD	4 α -phorbol 12,13-didecanoate
95% CI	95% confidence interval
Akt	protein kinase B
Ank	ankyrin
APACHE	Acute Physiology and Chronic Health Evaluation
APC	activated protein C
ATP	adenosine triphosphate
AUC	area under the curve
bAEC	bovine aortic endothelial cell
BCA	bicinchoninic acid
BK _{Ca} channel	large-conductance calcium-sensitive potassium channel
BL	baseline
BMDM	bone-marrow-derived macrophage
bp	base pair
BSA	bovine serum albumin
bTRPV1	bovine transient receptor potential vanilloid 1
Ca ²⁺ -CaM	calcium-calmodulin
Cap	capsaicin
cDNA	complementary deoxyribonucleic acid
cGMP	cyclic guanine monophosphate
CGRP	calcitonin gene related peptide
CI	cardiac index
CLP	cecal ligation and puncture

CO	cardiac output
C-terminus	carboxy terminus
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DRG	dorsal root ganglion
<i>E. coli</i>	<i>Escherichia coli</i>
EBM	endothelial basal medium
EC	endothelial cell
EDHF	endothelium-derived hyperpolarising factor
EDRF	endothelium-derived relaxing factor
EDTA	ethylenediaminetetraacetic acid
EETs	epoxyeicosatrienoic acid
EM	endothelium medium
eNOS	endothelial nitric oxide synthase
FBS	foetal bovine serum
Fura-2/AM	Fura-2-acetoxymethyl ester
sGC	soluble guanylyl cyclase
GSK	GSK1016790A
GTP	guanosine-5'-triphosphate
h	hour
HBSS	Hank's balanced salt solution
HC	HC-067047
HEK cell	human embryonic kidney cell
Het	heterozygous/heterozygote
HR	heart rate

hTRPV1	human transient receptor potential vanilloid 1
HUVEC	human umbilical vein endothelial cell
i.m.	intramuscular
i.p.	intraperitoneal
i.v.	intravenous
ICAM-1	intercellular adhesion molecule 1
ICU	intensive care unit
IK _{Ca} channel	intermediate-conductance calcium-sensitive potassium channel
IL-1	interleukin-1
IL-4	interleukin-4
IL-8	interleukin-8
iNOS	inducible nitric oxide synthase
IP ₃	inositol triphosphate
IVC	inferior vena cava
IVM	intravital microscopy
kb	kilobase
K _{Ca} channel	calcium-sensitive potassium channel
kDa	kilo Dalton
KO	knockout
L-NAME	N ^ω -nitro-L-arginine methyl ester
L-NMMA	N ^G -monomethyl-L-arginine
LPS	lipopolysaccharide
LSCI	laser speckle contrast imaging
MAP	mean arterial pressure
MCSF	macrophage colony-stimulating factor

min	minute
MLC	myosin light chain
MLCK	myosin light chain kinase
MLCP	myosin light chain phosphatase
MODS	multiple organ dysfunction syndrome
MRSA	methicillin-resistant <i>Staphylococcus aureus</i>
ms	millisecond
mTRPV1	murine transient receptor potential vanilloid 1
NA	noradrenaline
NAD ⁺ / NADH	nicotinamide adenine dinucleotide (oxidised and reduced forms respectively)
NGF	nerve growth factor
NIRS	near-infrared spectroscopy
NK ₁ receptor	neurokinin 1 receptor
NO	nitric oxide
NSAIDs	non-steroidal anti-inflammatory drugs
N-terminus	amino-terminus
OPS imaging	orthogonal polarisation spectral imaging
PAF	platelet aggregative factor
PBS	phosphate-buffered saline
PCO ₂	partial pressure of carbon dioxide
PCR	polymerase chain reaction
PCT	procalcitonin
PCV	packed cell volume
PGI ₂	prostacyclin
pH _i	intestinal pH

PI3 kinase	phosphoinositide 3-kinase
PIP ₂	Phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PLA ₂	phospholipase A ₂
PO ₂	qRT-PCR
Resus	resuscitation
RH-PAT	reactive hyperaemia peripheral arterial tonometry
RIPA	radioimmunoprecipitation assay
rpm	revolutions per minute
RR	ruthenium red
RT	room temperature
s	second
s.c.	subcutaneous
Sal	saline
SAPS	Simplified Acute Physiology Score
SDF imaging	sidestream dark field imaging
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SIRS	systemic inflammatory response syndrome
SK _{Ca} channel	small-conductance calcium-sensitive potassium channel
SO ₂	oxygen saturation
SOFA score	Sequential Organ Failure Assessment score

SP	substance P
SSC	Surviving Sepsis Campaign
SVR	systemic vascular resistance
TBS	tris-buffered saline
TBS-T	tris-buffered saline containing 1% Tween-20
TCA cycle	tricarboxylic acid cycle
TCO ₂	total carbon dioxide
TEA	tetraethylammonium
Temp	temperature
TLR4	toll-like receptor 4
TNF α	tumour necrosis factor α
TRP	transient receptor potential
TRPA	transient receptor potential ankyrin
TRPC	transient receptor potential canonical
TRPM	transient receptor potential melastatin
TRPML	transient receptor potential mucolipin
TRPP	transient receptor potential polycystin
TRPV	transient receptor potential vanilloid
UV	ultraviolet
Veh	vehicle
VGCC	voltage-gated calcium channel
VR-OAC	vanilloid receptor-related osmotically activated channel
VR1	vanilloid receptor 1
VSMC	vascular smooth muscle cell
w/v	weight/volume

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CHAPTER 1 | GENERAL INTRODUCTION

1.1 Introduction to sepsis

Sepsis is an overwhelming inflammatory response to infection that can progress to septic shock, characterised by refractory hypotension and insufficient organ perfusion. It is predominantly associated with nosocomial infections, and tends to occur in extremes of age, where it is associated with very high mortality rates. Sepsis and sepsis-associated multi-organ failure represent a tremendous healthcare burden and a significant cause of morbidity and mortality worldwide. Incidence has continued to increase over several decades (Melamed and Sorvillo, 2009), and septic shock is now the leading cause of death in intensive care units, accounting for an estimated 20,000 deaths per day worldwide (Daniels, 2011).

1.2 The global impact of sepsis

1.2.1 Incidence and epidemiology

Sepsis is one of the few conditions that is said to ‘strike with equal ferocity’ in developed and developing areas (Reinhart et al., 2013). Following a 1992 clinical consensus on the definition of sepsis (Bone et al., 1992b), comparable epidemiological studies have been carried out in almost all developed countries, and many developing countries. In developed nations, sepsis is thought to occur in 2% of all hospitalisations, and 6–30% of all ICU patients, with marked heterogeneity across different ICUs (Vincent et al., 2006).

A decade-long study of 343,860 admissions to 172 intensive care units in England, Wales and Northern Ireland between 1995 and 2005, found that 31,000 patients (9%) developed severe sepsis within 24 hours of admission; of these, 14,000 (45%) died in hospital (Harrison et al., 2006). Similar patterns have emerged in the USA (Martin et al., 2003, Dombrovskiy et al., 2007), Croatia (Degoricija et al., 2006), Australia and New Zealand (Finfer et al., 2004a).

In the US and EU combined, more than 1.5 million hospitalised patients are diagnosed with sepsis every year; approximately 15% go on to develop severe sepsis and septic shock, as defined in **Table 1** (Angus et al., 2001, Levy et al., 2010). Overall, mortality exceeds 40%, accounting for approximately 30% of all hospital-based deaths (Beale et al., 2009), making sepsis the leading cause of death in critically ill patients worldwide. This comprises estimated annual death figures of 36,800 in the UK (Daniels, 2011) and 215,000 in the USA, where sepsis is the 10th most common cause of death overall (Martin, 2012). Globally, sepsis claims 1000

lives every hour, or 24,000 deaths per day (Daniels, 2011). In developing countries, it accounts for 60–80% of childhood deaths, and affects 6 million infants every year (Reinhart et al., 2013).

Alarmingly, incidence of sepsis is still rising. A 20-year study of hospitalisations in the USA reported an 8.7% increase in the number of cases every year (Martin et al., 2003), and numerous reports from around the world have confirmed that global patterns of rising incidence are in excess of population growth (Martin et al., 2003, Dombrovskiy et al., 2007, Sundararajan et al., 2005, Dombrovskiy et al., 2005). A national hospital discharge survey conducted in the USA reported that hospitalisations for sepsis more than doubled between 2000 and 2008, though overall hospitalisations did not increase during this period (Hall et al., 2011).

There are several explanations for this rising incidence. Firstly, sepsis is a condition that tends to occur in extremes of age: as both life expectancy and the viability of pre-term babies grow, it is perhaps natural that global susceptibility to sepsis should increase in parallel. Demographic considerations are compounded by the escalating frequency of increasingly invasive clinical procedures, and, of course, the evolution of multi-drug-resistant pathogens (Ma et al., 2013b). The increasing prevalence of HIV infection may also contribute to greater susceptibility (Angus et al., 2001, Angus and Wax, 2001). In the developing world, poverty, malnutrition, limited access to vaccines and medical centres are all likely to play a significant role.

1.2.2 The economic burden of sepsis

In general, more than 70% of patients with severe sepsis will require intensive care - the most expensive form of hospital treatment. The overall yearly cost of sepsis treatment exceeds \$3.5 billion in the UK (Vincent et al., 2006) and \$16.7 billion in the USA (Angus et al., 2001). Between 1997 and 2008, the inflation-adjusted aggregate costs for treating hospitalised sepsis patients increased by an average 11.9% annually (HCUP, 2008). In spite of this increasing expenditure, sepsis is often fatal, and those who survive are more likely to have permanent organ damage, cognitive impairment, and physical disabilities.

1.2.3 The Surviving Sepsis Campaign

International guidelines for the management of severe sepsis were published in 2004 by the Surviving Sepsis Campaign (Dellinger et al., 2004) and were subsequently updated in 2008 (Dellinger et al., 2008). The guidelines contained specific Care Bundles created in collaboration with the Institute for Healthcare Improvement and designed to optimise and standardise

sepsis care around the world. In 2010, the SSC published results from its improvement programme. Data were reported for 15,022 patients from 165 centres across 30 countries; 71.5% presented with septic shock. Although absolute mortality was reduced by 5.4% over a 2-year period, in line with increasing compliance with SSC guidelines, reliability was not achieved, and compliance reached only 31% (Levy et al., 2010).

Although limited by voluntary data reporting, these results demonstrate that a multi-faceted and co-ordinated approach can be successful in changing treatment strategies for sepsis; compliance with guidelines increased significantly over time. Peak compliance was still only 31% at the end of the two-year period, however, and the SCC has further been subject to intense criticism over its links with industry during the second phase of implementation (Eichacker et al., 2006). This has largely centred on the inclusion of activation protein C (Xigris®; Eli Lilly and Co.) in the Management Care Bundle; Xigris® was subsequently withdrawn from the market in 2011, as discussed below. Debate surrounding the feasibility of complying with the more invasive aspects of care (especially the administration of early goal-directed therapy; EGDT) has also been fierce. Many tasks recommended by the Bundle are particularly invasive and require specialist skills and resources that are unavailable outside extremely well-resourced units (Daniels, 2011) and are frequently inaccessible in the UK (Sivayoham, 2007).

Nonetheless, SSC guidelines continue to be endorsed by the European Society of Intensive Care Medicine, the Intensive Care Society and the College of Emergency Medicine, among others.

1.3 Clinical diagnosis and treatment of sepsis

1.3.1 Signs and symptoms

Sepsis, or the systemic inflammatory response syndrome (SIRS) that occurs during infection (Bone et al., 1992a), is identified on the basis of numerous clinical symptoms in the presence of an established or presumed infection.

Definitions, signs and symptoms pertaining to the clinical diagnosis of sepsis are outlined in **Table 1**.

As described in **Table 1**, a clinical diagnosis of sepsis is made when a patient exhibits two or more SIRS criteria in the presence or suspected presence of microbial infection. Syndrome progression can result in severe sepsis, characterised by evidence of organ dysfunction, such as hypotension, oliguria, lactic acidosis or unexplained changes in mental state. Subsequent development of persistent hypotension that is refractory to fluid resuscitation is termed septic shock (King, 2007). In a meta-analysis of US hospital admissions, Martin et al. found that the primary sites of infection were the upper respiratory tract (40–44% of cases), genitourinary infections (9–18%) and intra-abdominal infections (9–14%) (Martin et al., 2003).

Rough estimates for the mortality rates associated with each stage of the sepsis syndrome are as follows (Martin, 2012):

- Sepsis: 10–20%
- Severe sepsis: 20–50%
- Septic shock: 40–80%

Difficulties in diagnosis arise from the lack of specificity and sensitivity of symptoms: SIRS symptoms may also occur in patients with pancreatitis, severe burns or major tissue trauma, for example (Reinhart, 2001). Thus, identification of specific biomarkers is essential for accurate diagnosis, appropriate intervention, and assessment of prognosis.

Systemic inflammatory response syndrome (SIRS): presence of two or more of the following clinical signs:

Core temperature $>38^{\circ}\text{C}$ or $<36^{\circ}\text{C}$

Heart rate > 90 beats/min

Respiratory rate >20 breaths/min, or arterial partial pressure of CO_2 (Pa_{CO_2}) <32 mmHg

White blood cells $>12,000/\text{mm}^3$, or $<4,000/\text{mm}^3$, or $>10\%$ immature (band) forms

Sepsis: SIRS with evidence or suspected evidence of infection

Severe sepsis: sepsis with evidence of end-organ dysfunction:

Decreased platelet count

Hypotension or hypoperfusion

Lactic acidosis

Oliguria

Acute changes in mental status

Abdominal pain

Mottled skin

Cardiac dysfunction, measured by echocardiography

Septic shock: Severe sepsis that is refractory to fluid resuscitation

Multiple organ dysfunction syndrome (MODS): dysfunction of multiple organs requiring fluid, vasopressor therapy and positive inotropic support.

Table 1. Definitions pertaining to the clinical diagnosis of sepsis. Adapted from Box 1 of Sepsis in Critical Care, J.E. King (2007) Critical Care Nursing Clinics of North America. (King, 2007)

1.3.2 Identification of sepsis

1.3.2.1 Microbial cultures

Following proliferation at the nidus of infection, microorganisms may invade the bloodstream directly, leading to a positive microbial culture from blood, urine, cerebrospinal or bronchial fluid – the most definitive method of diagnosis in sepsis. In some cases, however, locally proliferating microorganisms can release microbial products or toxins into the systemic circulation, triggering SIRS in the absence of a positive culture. A meta-review of diagnostic data found that a positive culture was confirmed in 8–73% of neonatal diagnoses, and in 8–88% of adult diagnoses (Carrigan et al., 2004). In a large-scale epidemiological analysis of hospital records in the USA between 1979 and 2000 (in which pathogens were identified in 51% of cases), Martin and colleagues found that 52.1% of cases were caused by gram-positive infection; 37.5% by gram-negative infection; 4.7% by polymicrobial infection; 4.6% by fungal infection; and 1.0% by anaerobic bacterial infection (Martin et al., 2003).

Gram-positive infections increased at a rate of 26.3% per year over the study period. This was attributed to a rise in nosocomial infections acquired following catheterisation or immunosuppressive therapy, for example, and is particularly alarming when viewed in the context of the rising prevalence of multi-drug-resistant pathogens; methicillin-resistant *Staphylococcus aureus* (MRSA) isolates range from 29–45%, with incidence rising continuously (Carrigan et al., 2004).

1.3.2.2 Clinical severity classification systems

Classification systems were devised to aid clinical diagnosis in the absence of a positive microbial culture. In addition to SIRS, the Acute Physiology Age and Chronic Health Examination (APACHE) and Simplified Acute Physiology Score (SAPS) have been used to stratify suspected sepsis cases based on various physiological indices (Carrigan et al., 2004). While such classification systems have been found to be useful in some studies (Oberholzer et al., 2001, Wanner et al., 2000), neither APACHE II (Balc et al., 2003, Muller et al., 2000, Giamarellos-Bourboulis et al., 2002, Oberhoffer et al., 1999b, Tugrul et al., 2002) nor SAPS II (Harbarth et al., 2001, Suprin et al., 2000) has proved sufficiently sensitive to distinguish between SIRS and sepsis in the majority of clinical studies.

1.3.2.3 Lactate

Under anaerobic conditions, pyruvate accumulates in the cytosol due to inhibition of the tricarboxylic acid (TCA) cycle. During anaerobic glycolysis, NAD^+ is reduced to NADH, and in order to regenerate NAD^+ , electrons donated by NADH are used to convert pyruvate into lactate via the lactate dehydrogenase enzyme (Fink, 1997). Thus, lactate accumulation is a characteristic feature of anaerobic metabolism, and is frequently observed in patients with septic shock. While this increase in blood lactate levels was traditionally considered to reflect regional dysoxia and anaerobic cellular metabolism, direct sepsis-mediated production, and alterations in clearance mechanisms, can complicate clinical interpretation of lactate measurements (Bakker, 2001).

Numerous clinical studies have indicated that lactate levels may have prognostic value in sepsis (Trzeciak et al., 2007a, Goyal et al., 2010), though they may be more useful when measured serially to guide response to therapeutic intervention (Nguyen et al., 2004). Nonetheless, there is considerable debate surrounding the validity of lactate measurements and their interpretation in this context. Hyperlactataemia is not always evident in septic patients (Hernandez et al., 2012a), and it was not considered in a 2010 SCC report to correlate with outcome (Levy et al., 2010). This is likely to reflect the low specificity of lactate for organ hypoperfusion secondary to severe sepsis.

Interestingly, hyperlactataemia with normal blood pressure has been observed in up to 25% of septic patients (Daniels, 2011). These normotensive patients – termed ‘cryptic shock’ patients – exhibit no difference in mortality to those with overt shock (Puskarich et al., 2011), indicating that haemodynamic indices alone are not sufficiently indicative of regional hypoxia or indeed prognosis.

1.3.2.4 Procalcitonin

While a vast range of biomarkers (from C-reactive protein to circulating cytokines and adhesion molecules) have been trialled as prognostic markers in sepsis, procalcitonin (PCT) has emerged as perhaps the most specific marker of bacteraemia (the most common cause of sepsis), though its induction in fungal and viral infections is more variable (Beaune et al., 1998, Hammer et al., 1998, Dornbusch et al., 2005, Assicot et al., 1993).

A propeptide of calcitonin, PCT is produced in parafollicular cells of the thyroid gland (Snider et al., 1997). Under physiological conditions, circulating levels of procalcitonin are extremely low (>0.1 ng/ml). In sepsis, however, PCT is produced in large quantities by extrathyroid tissues –

predominantly leukocytes and to a lesser extent the liver (Oberhoffer et al., 1999a) – with plasma concentrations sometimes exceeding several hundred ng/ml (Brunkhorst et al., 1998). Its role in sepsis pathogenesis is not clear, though administration of PCT has been shown to increase mortality, and neutralisation to confer significant protection in a murine model of sepsis (Nylen et al., 1998).

Use of PCT as a biomarker for sepsis diagnosis, prognosis and antibiotic efficacy confers numerous advantages over the use of other clinical biomarkers: in contrast to cytokines, its long half-life of 22–29h (Assumma et al., 2000), and its prolonged increase during sepsis (Dandona et al., 1994) are favourable for clinical measurement. It is detectable in plasma approximately 20 h earlier than C-reactive protein (Reinhart, 2001), and its stability at room temperature negates the need for cold storage of samples (Carrigan et al., 2004).

Although PCT levels have been shown in numerous studies to correlate with infection severity and outcome, conferring greater sensitivity than alternative biomarkers (Assicot et al., 1993, Azevedo et al., 2012, Boeken et al., 1998, Reith et al., 1998, Bell et al., 2003, Matesanz et al., 2003, Balc et al., 2003, Kocabas et al., 2007, Rau et al., 2007, Aikawa et al., 2005, Endo et al., 2008, Meisner et al., 1999, Oberhoffer et al., 1999b), other studies have found no diagnostic advantage (Ruokonen et al., 2002, Zhao et al., 2013, Oliveira et al., 2013, Wanner et al., 2000, Giamarellos-Bourboulis et al., 2002, Tugrul et al., 2002, Ugarte et al., 1999, Hensler et al., 2003). The usefulness of PCT as a sepsis biomarker is further confounded by evidence that its ability to differentiate between SIRS and sepsis is dependent on patient age (Carrigan et al., 2004), and furthermore that it may be produced in some cases of non-infectious inflammation (Monneret et al., 1998). Additionally, the diagnostic and prognostic value of PCT measurement may be diminished in leukopaenic patients, who produce significantly lower concentrations of PCT, even during severe sepsis and septic shock (Svaldi et al., 2001).

1.3.2.5 microRNAs

Despite some evidence of the diagnostic and prognostic value of the biomarkers described above, incidence of sepsis-associated mortality is high and rising; it is clear there is an urgent unmet healthcare need for early, sensitive and specific biomarkers to aid rapid and accurate diagnosis. Furthermore, delays associated with conventional biomarker analysis, and issues of sensitivity and specificity often result in the use of broad-spectrum antibiotics and inappropriate deployment of expensive technology, without significant benefit to septic patients (Dellinger et al., 2004, Gaieski et al., 2010). Not only does this add considerably to the

economic burden of sepsis, it is also particularly worrying in the context of rising antibiotic resistance.

In recent years, microRNAs – or non-coding RNAs – have emerged as important post-translational regulators of genes linked to inflammation and immunity, among myriad other cellular processes (Baltimore et al., 2008, O'Connell et al., 2010). Induction or suppression of specific microRNAs in pathological conditions has already led to the quantification of expression levels for diagnostic and prognostic purposes in numerous cancers (Ji et al., 2009, Calin and Croce, 2006). Recently, this approach has also been applied to the study of inflammation and sepsis, revealing altered expression of a limited set of specific microRNAs both in vitro (O'Connell et al., 2007, Tili et al., 2007) and in vivo (Vasilescu et al., 2009, Wang et al., 2010).

In a ground-breaking study published in 2013, Ma and colleagues undertook massive parallel sequencing of the entire whole blood microRNAome of patients with SIRS, sepsis or no obvious pathology, from two independent cohorts. Two microRNAs – miR-150 and miR-4772-5p-iso – were found to discriminate between the three clinical conditions, with 90.5% specificity and 81.8% sensitivity in distinguishing sepsis from SIRS. Overall, 86% diagnostic accuracy was established across both cohorts, and this was later validated in vitro; pharmacological activation of toll-like receptors in isolated peripheral blood monocytes was sufficient to upregulate miR-4772-5p-iso within 24 h. Measurement of these microRNAs in SIRS patients may help to facilitate rapid identification of sepsis patients and optimise antimicrobial intervention.

1.3.3 Current lines of treatment

There are currently no specific anti-sepsis drugs available for clinical use, following the withdrawal of Xigris® by Eli Lilly & Co. in 2011. The first-line of treatment for septic patients is rapid administration of antimicrobial agents and intensive goal-directed fluid resuscitation. In cases of septic shock, where patients exhibit profound hypotension in spite of fluid resuscitation, vasopressors (noradrenaline in the first instance) may be indicated, either alone or in combination with inotropic support, transfusion of blood products or steroid therapy (Fitch and Gossage, 2002).

1.3.3.1 Antimicrobial therapy

Although a definitive source of infection is only identified in up to 80% of patients with severe sepsis (Wheeler and Bernard, 1999), administration of antibiotics is one of the only interventions known to reduce sepsis-associated mortality (Wheeler and Bernard, 1999, Fitch and Gossage, 2002). The choice of antibiotics is generally based on the patient's history and hospital prevalence and susceptibility patterns, and broad-spectrum antibiotics are often used if specific pathogens have not been identified (Merx and Weber, 2007). Expedient administration is critical: for every 1 h delay in the administration of antibiotics to patients with septic shock, chances of survival are decreased by almost 8% (Gaieski et al., 2010). A 2010 report detailing the results of the SSC initiative, found that early administration of antibiotics and sampling for microbial culture prior to administration were each independently associated with survival over the first 24 h [odds ratios (OR) for mortality 0.86 and 0.76, respectively; upper 95% CI limits < 1.0, $p < 0.001$, for each] (Levy et al., 2010).

1.3.3.2 Fluid resuscitation

In a seminal 2001 study, Rivers and colleagues demonstrated that early, aggressive, goal-directed fluid resuscitation significantly improved survival in patients with severe sepsis or septic shock (Rivers et al., 2001). This intervention was found to optimise cardiac preload and afterload, and to improve cardiac contractility. Subsequently, fluid resuscitation has been shown to improve microcirculatory perfusion and survival in septic patients (Trzeciak et al., 2008). Similar improvements in cardiovascular performance and survival have been demonstrated in murine models of resuscitated sepsis (Hollenberg et al., 2001, Miyaji et al., 2003), as well as rats (Anning et al., 2004, Smith et al., 1993), pigs (Wu et al., 2013, Correa et al., 2012, Marx et al., 2004), dogs (Natanson et al., 1990) and sheep (Ertmer et al., 2011).

Resuscitation with excessively high volumes of fluid, however, has also been associated with increased mortality in a porcine model of endotoxaemia (Brandt et al., 2009). Furthermore, although continuous infusion with lactated Ringer's solution improved cardiac index and systemic oxygen delivery after 165 min in a canine model of bacteraemia, laser Doppler flowmetry and gut tonometry revealed no significant improvement in mesenteric oxygen delivery, oxygen extraction ratios or portal lactate at the end of the study (Lagoa et al., 2004).

Nonetheless, early fluid resuscitation remains a cornerstone of sepsis treatment, and forms a critical component of the SSC guidelines (Levy et al., 2012). Much debate has surrounded the optimal nature of the resuscitation fluid, though evidence suggests that crystalloid fluids

achieve a similar outcome to the more expensive colloid agents, such as albumin or Hetastarch (Finfer et al., 2004b, Hollenberg et al., 2004).

1.3.3.3 Glycaemic control

In 2001, van den Berghe and colleagues demonstrated that maintenance of blood glucose at 4.4–6.1 mmol per litre resulted in lower morbidity and mortality among critically ill patients than did conventional therapy that maintained blood glucose at 10–11.1 mmol per litre (van den Berghe et al., 2001). Patients with bacteraemia who were treated with intensive insulin therapy had lower mortality than those receiving standard therapy (12.5% v. 29.5%) regardless of their diabetic status. The molecular mechanisms underlying this protective effect have not been established, though the phagocytic function of neutrophils is known to be impaired in conditions of hyperglycaemia (Van Oss, 1971). Insulin is also thought to confer antiapoptotic and cardioprotective effects by stimulating nitric oxide production via PI3 kinase-Akt-mediated phosphorylation of endothelial nitric oxide synthase (eNOS) (Gao et al., 2002). SSC results showed that tight glycaemic control was independently associated with survival (Levy et al., 2010); continuous monitoring of blood glucose is essential, however, to avoid complications of brain hypoglycaemia.

1.3.3.4 Pharmacological treatment

Myriad agents, including vasopressors, inotropes, anti-inflammatory agents and anti-coagulants have been trialled in the treatment of sepsis. Where hypotension is refractory to fluid resuscitation, noradrenaline and dopamine are the initial vasopressors of choice, and may be administered alone or in combination with dobutamine (if cardiac output is depressed), transfusion of blood products or steroid therapy (Dellinger et al., 2008). The use of corticosteroids is controversial, however, as some studies indicate that they do not improve survival (Bone et al., 1987) and may worsen outcomes by increasing the frequency of secondary infections (Cronin et al., 1995). A 2001 study by Annane, on the other hand, indicated that patients with persistent shock requiring vasopressors and prolonged mechanical ventilation may benefit from low doses of corticosteroids (Annane, 2001), since such patients may have relative adrenal insufficiency (Shenker and Skatrud, 2001). Catecholamines increase arterial pressure through activation of vascular adrenergic receptors, which may be downregulated in sepsis; corticosteroids increase the expression of adrenergic receptors, however, which may explain their efficacy in patients with persistent shock (Shenker and Skatrud, 2001).

1.3.3.5 An unmet clinical need

In spite of SSC treatment guidelines (Dellinger et al., 2008), death rates from sepsis remain stubbornly high, and it is clear that more specific and effective therapies are necessary to tackle this burgeoning healthcare problem. A greater understanding of the pathophysiological mechanisms underlying sepsis mortality, and the development of more relevant pre-clinical models in which to test novel interventions are of paramount importance.

1.4 The cardiovascular pathogenesis of sepsis

The pathogenesis of sepsis has traditionally been ascribed to an inappropriate and overwhelming inflammatory response. This theory was popularised by Lewis Thomas, who in 1972 wrote:

'The micro-organisms that seem to have it in for us in the worst way — the ones that really appear to wish us ill — turn out on close examination to be rather more like bystanders, strays, strangers in from the cold. They will invade and replicate if given the chance, and some of them will get into our deepest tissues and set forth in the blood, but it is our response to their presence that makes the disease. Our arsenals for fighting off bacteria are so powerful, and involve so many different defense mechanisms, that we are in more danger from them than from the invaders.'

(Thomas, 1972)

Over the last few decades, however, this view has begun to be challenged and may be overly simplistic: clinical trials of anti-inflammatory and immunomodulatory drugs have largely failed, and while circulating cytokines undoubtedly have a role in pathogenesis, blocking them entirely actually increases mortality, both in pre-clinical models of sepsis (Eskandari et al., 1992, Echtenacher et al., 2001) and in human patients (Fisher et al., 1996, Fisher et al., 1994). Interestingly, C3H/HeJ mice, which are resistant to endotoxin due to a mutation in the TLR4 gene, have increased mortality in sepsis (Hagberg et al., 1985, Hotchkiss et al., 1999). Although TLR4 activation can undoubtedly produce deleterious effects, blocking it entirely may be detrimental, and trials of monoclonal anti-endotoxin antibodies have failed (Warren et al., 1993). Furthermore, the fundamental differences between the immune systems of pre-clinical species and humans (Seok et al., 2013) limit meaningful pre-clinical identification of immunological targets.

The essential and beneficial role of cytokines in mediating defence against infections is underscored by the observation that sepsis frequently develops in patients treated with antagonists of tumour necrosis factor alpha (TNF α) for rheumatoid arthritis (Keane et al., 2001). As a consequence of this, research focus has begun to shift away from the immune response towards other physiological systems, most notably the cardiovascular system – often affected in sepsis and always compromised in septic shock. This intense research interest may have been fuelled by the observation that the presence of cardiovascular dysfunction in sepsis increases mortality rates from approximately 20% to 70–90% (Parrillo et al., 1990).

1.4.1 A historical perspective

The first descriptive account of sepsis-associated cardiovascular dysfunction in humans was provided in 1951 by Burton Waisbren, who described two subpopulations of septic patients: the first exhibited hypotension with bounding pulses, fever, oliguria and flushing; the second, hypotension with pallor, clamminess and low-volume pulses (Waisbren, 1951). These distinct subpopulations are now thought to represent varying degrees of volume resuscitation; aggressive fluid administration is associated with a hyperdynamic state that is not observed in inadequately resuscitated patients (Merx and Weber, 2007).

Over the 60 years since Waisbren's initial report, advancements in technology have facilitated more detailed and quantitative assessment of haemodynamic dysfunction in sepsis, allowing ICU clinicians and pre-clinical scientists to begin probing the molecular underpinnings of disease progression. The cardiovascular derangements associated with sepsis progression are shown schematically in **Figure 1**.

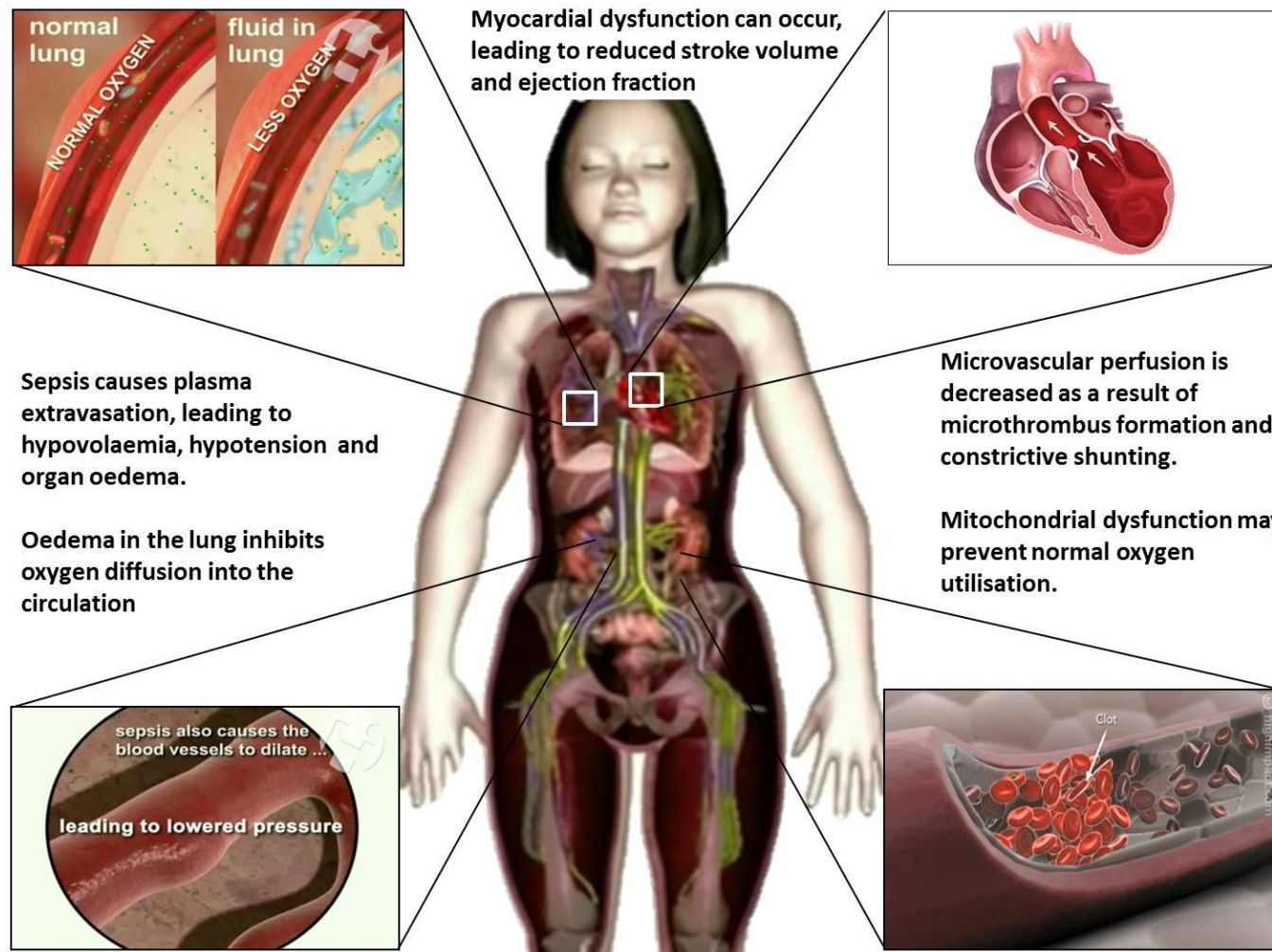


Figure 1. Cardiovascular derangements associated with sepsis progression. Figure adapted from 'Sepsis', Medical Animations by High Impact Graphics (HighImpactGraphics, 2011).

1.4.2 Molecular mechanisms of sepsis-associated cardiovascular dysfunction

Release of bacterial products into the systemic circulation causes activation of immune cells – predominantly macrophages – via toll-like receptors on the cell surface. Fungal and viral infections induce a similar response, via different molecular pathways. Receptor stimulation triggers the release of inflammatory mediators, predominantly interleukin-1 (IL-1) and TNF α , the primary cytokines implicated in the early stages of sepsis. These cytokines act synergistically to activate endothelial cells, smooth muscle cells and leukocytes, stimulating production of secondary mediators, including inducible nitric oxide synthase (iNOS), platelet aggregating factor (PAF), prostaglandins, leukotrienes and interleukins, which combine to produce significant cardiovascular, circulatory and haematological effects.

Of all the secondary mediators induced during sepsis, iNOS has received particularly intensive research attention, both in the laboratory and in the clinic. Under physiological conditions, nitric oxide (NO) is produced in the endothelium by a constitutive endothelial (e) isoform of the NOS enzyme. This physiological production of NO, in the picomolar range, is essential for regulation of blood pressure and blood flow distribution, and is transient in nature, with a critical dependency on calcium transients (Kirkeboen and Strand, 1999). Induction of the high-output iNOS isoform in endothelial and smooth muscle cells, as well as leukocytes, however, results in overproduction of NO, in the nanomolar range, and being independent of calcium transients, iNOS activity can be sustained for up to several days (Kirkeboen and Strand, 1999). While essentially a protective antimicrobial response, prolonged upregulation of iNOS can have severe vasoregulatory, myocardial and cytotoxic consequences.

The vasoregulatory function of NO hinges predominantly on its activation of the soluble heme-containing enzyme guanylate cyclase (GC), which catalyses the production of 3'-5'-cyclic monophosphate (cGMP). cGMP stimulates vascular smooth muscle relaxation through inhibition of calcium entry, and activation of hyperpolarising K⁺ currents and protein kinase G (PKG), which in turn activates myosin light chain phosphatase, leading to dephosphorylation of myosin light chains and smooth muscle relaxation (**Fig. 2**) (Derbyshire and Marletta, 2012). Thus, excessive production of NO induces profound systemic vasodilatation – a hallmark of sepsis and septic shock.

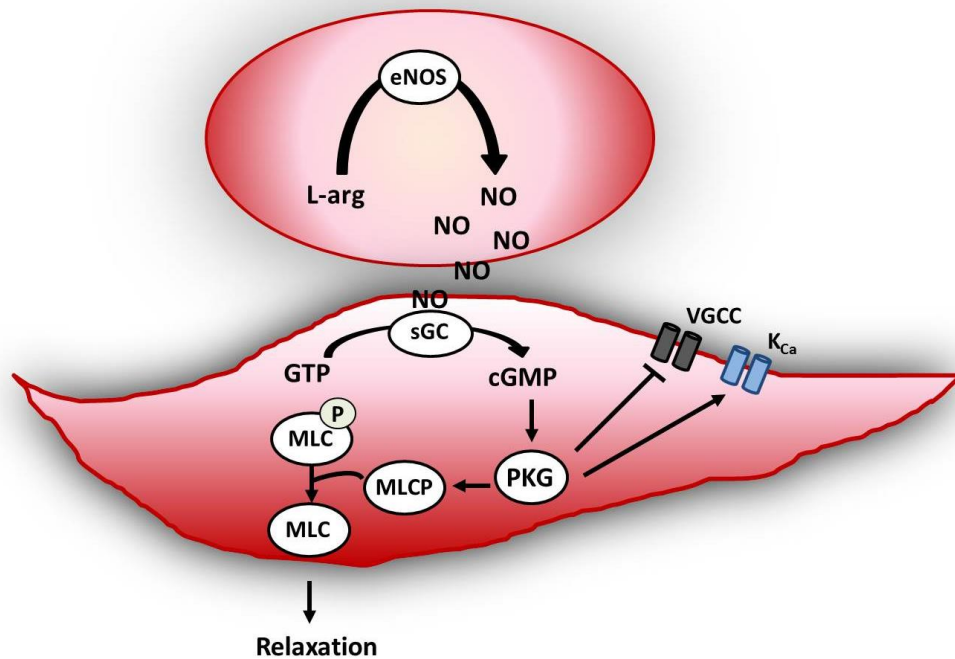


Figure 2. Mechanism of NO-dependent vasodilatation. NO is synthesised from its L-arginine (L-Arg) precursor by eNOS. NO is a gaseous mediator that can diffuse to underlying smooth muscle cells to activate soluble GC, which in turn catalyses the production of cGMP. cGMP elicits vasodilatation through inhibition of voltage-gated Ca²⁺ channels (VGCC); activation of Ca²⁺-activated K⁺ channels (K_{Ca}); and activation of myosin light chain phosphatase (MLCP), which dephosphorylates myosin light chains (MLC) leading to smooth muscle relaxation.

In addition to its vasoregulatory functions, NO also regulates numerous other cardiovascular and immune processes. Importantly, under oxidative conditions (as encountered in sepsis, where reactive oxygen species are generated in large quantities by inflammatory cells), NO can react with superoxide molecules to form highly cytotoxic peroxynitrite radicals (Liaudet et al., 2013). As a consequence of its involvement in haemodynamic, cardiac and homeostatic regulation, the role of NO in sepsis has been studied intensively for several decades. Translation of this research into clinical practice has largely failed, however, as discussed in section 1.5.

1.4.3 Global haemodynamics in sepsis

1.4.3.1 Haemodynamic alterations in sepsis

Sepsis, which can progress to septic shock, is characterised by significant haemodynamic alterations. The haemodynamic profile of sepsis is phasic in nature, and depends critically on the level of clinical intervention. An initial hypodynamic phase arises in parallel with immune activation; early immune mediator release increases vascular permeability to allow leukocyte transmigration to sites of infection (Bridges and Dukes, 2005). As a result, intravascular fluid leaks into the interstitial space, resulting in hypovolaemia. This, in combination with the profound decrease in systemic vascular resistance (SVR) elicited by mediators such as NO and PAF, precipitates a substantial drop in blood pressure (Trzeciak and Rivers, 2005).

Following adequate intravascular fluid resuscitation, a tachycardic response to hypotension is initiated, leading to elevated cardiac output, characteristic of the hyperdynamic phase of sepsis (Bone, 1991). This increase in cardiac output can mask the myocardial dysfunction that also occurs as sepsis progresses, however; Parker and colleagues were among the first to demonstrate significantly diminished stroke volume and ejection fraction in septic patients with normal cardiac output (Parker et al., 1984). Several mechanisms are postulated to contribute to sepsis-associated myocardial dysfunction, including iNOS-mediated formation of cytotoxic peroxynitrite radicals, and NO- and cytokine-mediated inhibition of responsiveness to catecholamines (Preiser et al., 2001, Khadour et al., 2002).

Haemodynamic monitoring is now routine clinical practice in critical care wards, and measurement of mean arterial pressure (MAP) has been a mainstay of patient evaluation for decades. The diagnostic and prognostic usefulness of this parameter is increasingly called into question, however. A seemingly normal blood pressure may mask significant haemodynamic suppression in a normally hypertensive individual, for example, if patient history is not known. Furthermore, it is now known that arterial blood pressure does not reflect regional blood flow and tissue oxygenation, which are more sensitive indicators of disease severity; MAP may be

maintained by compensatory mechanisms, even when organ perfusion is severely compromised (Ahrens, 2006). There is now a growing consensus that blood pressure should be measured as a secondary parameter, and that measurements of tissue oxygenation and blood flow, particularly at the microcirculatory level, confer greater diagnostic and prognostic relevance.

1.4.3.2 Global haemodynamics as a primary endpoint in sepsis

Under physiological conditions, the relationship between global haemodynamics and microcirculatory flow is relatively loose, owing to the intrinsic myogenic properties of vascular smooth muscle (De Backer et al., 2010). Increases in arterial pressure are buffered by a myogenic constrictor response in small arteries and arterioles that ensures constant blood flow in microvascular beds (Davis and Hill, 1999). Correspondingly, decreases in arterial pressure are buffered by loco-regional vasodilatory responses. Nonetheless, it is reasonable to assume the existence of a critical threshold, below which organ perfusion becomes pressure-dependent: microcirculatory flow would certainly also cease with complete circulatory arrest. It is conceivable that microthrombus formation and regional imbalances between vasoconstrictor and vasodilatory stimuli could drive up this autoregulatory threshold and that pressure-oriented therapy could correspondingly improve organ perfusion. Clinical evidence overwhelmingly refutes this hypothesis, however.

In 1995, Gattinoni and colleagues demonstrated that goal-oriented haemodynamic therapy (aimed at elevating either cardiac index or systemic oxygen delivery) did not improve mortality in septic patients (Gattinoni et al., 1995). Since then, other studies have shown that pressure-oriented therapy does not alter cutaneous microcirculatory perfusion, markers of splanchnic perfusion, uresis or markers of kidney dysfunction (LeDoux et al., 2000, Bourgoin et al., 2005). In 2009, two studies investigating the impact of incrementally elevating MAP through noradrenaline infusion concluded that changes in arterial pressure had no effect on sublingual microvascular blood flow and regional oxygen delivery (Jhanji et al., 2009, Dubin et al., 2009). These latter observations may explain the failure of haemodynamic-oriented therapy to improve survival in septic patients (Gattinoni et al., 1995), suggesting that global haemodynamic performance may not be particularly indicative of disease progression, since it does not appear to reflect perturbations at the microvascular level. Furthermore, dobutamine has been shown to confer independent effects on microcirculatory function and MAP (Hernandez et al., 2013, De Backer et al., 2006).

1.4.4 Microcirculatory dysfunction in sepsis

Historically, critical care of septic patients, and evaluation of novel interventions in pre-clinical models of sepsis have centred on achieving target blood pressures and normalising global haemodynamic parameters, as discussed previously. In 2002, however, De Backer and colleagues were among the first to demonstrate directly significant alterations in microcirculatory perfusion in patients with severe sepsis or septic shock (De Backer et al., 2002). Since then, sepsis has increasingly come to be understood as a ‘disease of the microcirculation’ (Spronk et al., 2004) – a paradigm that is gaining considerable traction in clinical research, but is not as frequently addressed in pre-clinical studies, as shown in **Figure 3**.

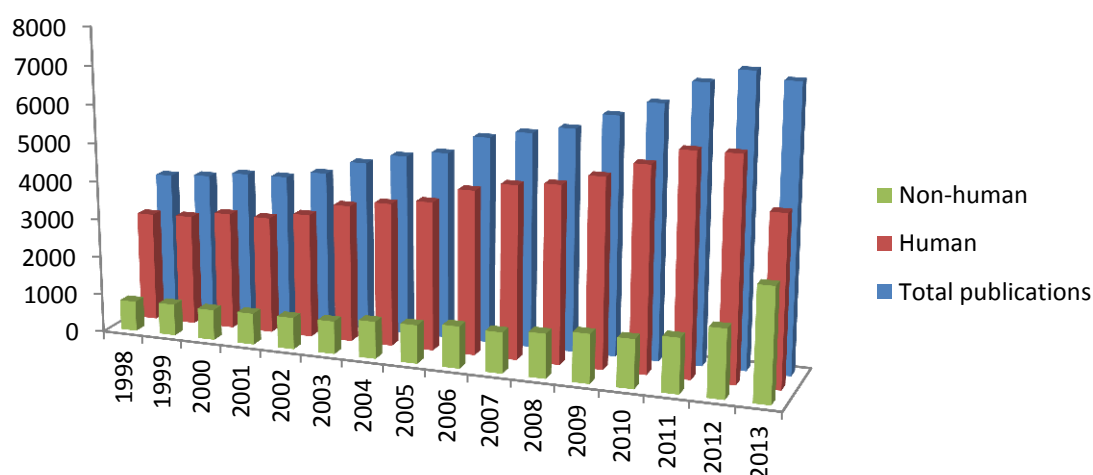


Figure 3. Number of publications relating to the microcirculation in sepsis and its sequelae since 1998. Source: PubMed; search term “microcirculation” AND “sepsis” OR “severe sepsis” OR “septic shock” OR “endotoxemia” OR “endotoxaemia”, either unlimited, limited to human data or limited to non-human data.

1.4.4.1 The oxygen extraction deficit

Following the failure of haemodynamic-oriented treatment regimens to improve mortality in septic patients (Gattinoni et al., 1995), it has become increasingly clear that maintaining systemic blood pressure is often associated with inadequate organ perfusion, and that stabilisation of the former may occur at the expense of the latter (Sakr et al., 2004, Spronk et al., 2004). Indeed, indicators of regional oxygenation, such as gastric pH, lactate and acid-base balance often remain abnormal despite the restoration of systemic oxygen delivery, and multiple organ failure commonly occurs in septic patients even after a stable haemodynamic status has been re-established (De Backer et al., 2002). This so-called 'oxygen extraction deficit' has been the subject of intense debate and investigation for many years, and is undoubtedly the result of dysfunction at the site of tissue oxygen exchange; the relative contributions of direct cellular impairment and/or redistribution of microcirculatory blood flow, however, remain incompletely understood.

1.4.4.2 Mechanisms of microcirculatory dysfunction in sepsis

1.4.4.2.1 Cytopathic hypoxia

Mitchell Fink was among the first to suggest that sepsis pathogenesis was not a problem of insufficient oxygen delivery, but rather one of impaired oxygen utilisation as a result of mitochondrial dysfunction – a phenomenon he termed 'cytopathic hypoxia' (Fink, 1997). This conclusion was drawn from previously published observations that in a rat model of cecal ligation and puncture, systemic oxygen delivery and partial pressure of oxygen (PO₂) in skeletal muscle were both severely compromised; administration of albumin solution (to reverse hypovolaemia) restored both parameters, but did not affect the decrease in tissue ATP levels, or the increase in tissue lactate levels observed in un-resuscitated rats (Astiz et al., 1988). Similar effects had been observed in canine (Vallet et al., 1994) and porcine (Hasibeder et al., 1996) models of endotoxaemia, where intestinal mucosal PO₂ remained low despite restoration of mesenteric oxygen delivery.

There is also *in vitro* evidence to support the theory of cytopathic hypoxia: endotoxin has been found to reduce oxygen consumption in cultured hepatocytes (Rosser et al., 1998) and erythrocytes (King et al., 1999) despite an adequate supply of oxygen in the culture medium. Other studies suggest that mitochondrial dysfunction is not intrinsic to sepsis, however, and is merely the result of tissue hypoxia (Fry et al., 1979, Geller et al., 1986). The development of mitochondrial depression in septic patients, and its relation to microcirculatory failure and localised tissue hypoxia, is still not well understood.

1.4.4.2.2 Arteriovenous shunting in sepsis

An alternative (or perhaps an adjunct) to the 'cytopathic hypoxia theory' (Fink, 1997) is the 'shunting theory of sepsis' put forward by Ince and Sinaasappel in 1999. According to this theory, oxygen transport is shunted directly from the arterial to the venous system in sepsis, bypassing microcirculatory units, and accounting for the oxygen extraction deficit observed clinically (Ince and Sinaasappel, 1999). This regional hypoxia was also thought to contribute to the mitochondrial dysfunction described by Fink (Fink, 1997).

In order to preserve oxygen delivery to critical vascular beds in the brain and myocardium, compensatory shunting mechanisms are initiated under pathological conditions, which favour derecruitment of compliant beds in the skin and splanchnic areas, for example. While this is a vital defence mechanism to preserve heart and brain tissue in acute hypovolaemia, prolonged microcirculatory shutdown, in the splanchnic region in particular, inevitably leads to hypoxic tissue injury and the development of multiple organ failure (Takala, 1996, Spronk et al., 2004). Thus, a process that was initiated as part of an adaptive mechanism may rapidly become maladaptive.

According to the shunting theory of sepsis, recruitment of shunted microcirculatory units could be expected to confer significant therapeutic benefit. Correspondingly, a meta-analysis published by Buwalda and Ince in 2002 demonstrated that use of vasodilators to open the microcirculation and promote microcirculatory flow improved tissue perfusion and oxygen extraction both in numerous animal models and in septic patients (Buwalda and Ince, 2002). Furthermore, a gap between microcirculatory PO_2 and venous PO_2 (which would widen if oxygen-rich arteriolar blood were shunted directly to the venous circulation) has been demonstrated in a porcine model of endotoxaemia (Ince and Sinaasappel, 1999), and in a porcine model of haemorrhagic shock (Sinaasappel et al., 1999), though the magnitude of the PO_2 gap was greater in the former model.

There are numerous mechanisms by which such shunting could occur. Convective arteriovenous shunting through anatomical anastomoses has previously been demonstrated in the rat, and may become increasingly active in sepsis (Menger and Vollmar, 1996). Alternatively, oxygen may diffuse directly between arterioles and venules in close proximity to one another, as observed in the heart under conditions of low coronary flow (Roth and Feigl, 1981). A third potential mechanism is known as 'vascular steal', whereby certain microvascular units 'steal' flow from others by selective vasodilatation (Shepherd and Kiel, 1992).

Microcirculatory heterogeneity is a key feature of sepsis (De Backer et al., 2002) and may be exaggerated in certain microvascular beds by microthrombus formation, decreased erythrocyte deformability, oedema formation and leukocyte sequestration. Finally, the PO₂ gap between the micro- and venous circulation may be explained by an insufficiently fast dissociation between oxygen and haemoglobin in microvascular beds during sepsis; at low saturation levels, the oxygen 'off-load time' for an erythrocyte can exceed its transit time in the microcirculation, such that excessive off-loading occurs in the venous circulation. (Gutierrez, 1986).

1.4.4.2.3 Other factors contributing to microcirculatory failure

In addition to arteriovenous shunting, several other mechanisms are thought contribute to microcirculatory collapse in sepsis: decreased erythrocyte deformability with consequently increased viscosity (Astiz et al., 1995); elevated numbers of activated neutrophils with decreased deformability and increased aggregability following upregulation of adhesion molecules (Linderkamp et al., 1998); activation of the clotting cascade with increased fibrin deposition and microthrombus formation (Diaz et al., 1998); and the failure of vasoautoregulatory mechanisms (Avontuur et al., 1997).

The balance between vasoconstrictive and vasodilatory substances may be altered in sepsis. Potent vasoconstrictors, such as endothelin, are upregulated under inflammatory conditions (Shindo et al., 1998), and may contribute to localised impairment of NO-mediated dilatation. This may be compounded by a reduction in NO availability, precipitated by uncoupling of iNOS, due to reduced availability of L-arginine substrate (Luiking et al., 2009) which favours the formation of peroxynitrite radicals. Moreover, degeneration of the glycocalyx – the layer that covers endothelial cells, and regulates adherence of circulating leukocytes – probably also play an important role in the development of microvascular dysfunction (Henrich et al., 2010).

1.4.4.2.4 The role of the endothelium

The endothelium is a critical regulator of vascular tone and permeability; its activation in sepsis precipitates both the inflammatory and coagulation cascades (Aird, 2003). Clinical evaluation of endothelial function is generally based on measurements of vasodilatation in response to pharmacological stimulation or changes in shear stress, though circulating surrogate markers of endothelial activation have often been measured in clinical studies of sepsis (Deanfield et al., 2007). This likely reflects the technical difficulty and impracticality of techniques such as laser Doppler flowmetry, plethysmography and flow-mediated dilatation of the brachial artery, (Davis et al., 2009). Studies in which the aforementioned techniques have been used, however,

have generally shown normal baseline blood flow but impaired reactive hyperaemic responses in septic patients, though these studies have been tended to be small, and have neglected to compare reactive hyperaemia with L-arginine or circulating markers of endothelial activation (Young and Cameron, 1995, Neviere et al., 1996, Kubli et al., 2003, Hartl et al., 1988, Astiz et al., 1995, Vaudo et al., 2008, Creteur et al., 2007). More recently, investigators using near-infrared spectroscopy (NIRS) have found impaired microvascular responsiveness in sepsis, though the nature of the relation between NIRS and endothelial NO activity is unclear (Creteur et al., 2007).

1.4.4.3 Evaluation of microcirculatory perfusion

Until relatively recently, visualisation of the microcirculation in human patients was conducted by intravital microscopy (IVM), and was restricted to very superficial beds in the eye or nailfold, owing to the bulky nature of the IVM equipment and the requirement for fluorescent dyes to visualise blood flow. The perfusion of internal organs was inferred from measurements of surrogate markers (using gut tonometry to measure intestinal gases, or blood tests to quantify levels of lactate and oxygen saturation, for example). The advent of orthogonal polarisation spectral (OPS) imaging in the late 1990s allowed clinicians to visualise directly the microcirculation of internal organs for the first time (Groner et al., 1999), and its use has been pivotal in establishing the defining role of microcirculatory dysfunction in sepsis pathogenesis.

OPS imaging is based on the illumination of tissue with linearly polarised green light. Contrast is generated by the absorption of the polarised light by haemoglobin in the blood; erythrocytes appear black against the white background of surrounding tissue, which reflects the incident light. OPS is highly sensitive to internal light scatter, however, which can cause blurring and limit visualisation of capillaries. Furthermore, its use in the clinic is limited by the requirement for high-powered and bulky light sources. More recently, sidestream dark field (SDF) imaging has been introduced (Goedhart et al., 2007). This modality, which operates on a similar but more refined basis, can be incorporated into a hand-held device, provides higher quality images, is less subject to movement and pressure artefacts, and allows more accurate quantification of microcirculatory flow.

In 2009, Davis et al. introduced a new technique – reactive hyperaemia peripheral arterial tonometry (RH-PAT) to measure microvascular endothelial function in a user-independent and bedside-compatible manner (Davis et al., 2009). It is at least 50% dependent on endothelial NO activity (Nohria et al., 2006), and uses finger probes to measure digital pulse wave amplitudes detected by a pressure transducer. The authors found that microvascular reactivity was impaired in sepsis and was further exaggerated in severe sepsis (Davis et al., 2009). Subjects

with severe sepsis were more likely to have endothelial dysfunction, even after correction for known associations and risk factors, such as smoking or diabetes.

In pre-clinical research, the ability to conduct more invasive intravital microscopy and laser Doppler flowmetry imaging has allowed investigators to assess internal microcirculatory flow in a variety of animal models of disease. SDF is also used pre-clinically in larger species, such as cows and sheep.

1.4.4.4 Microcirculatory perfusion as an endpoint in sepsis

A link between microcirculatory perfusion and sepsis severity was initially inferred from recordings of big toe temperature in septic patients, several decades ago (Joly and Weil, 1969). With the advancement of relevant technology, monitoring of microcirculatory perfusion in the ICU is becoming increasingly common and has led to substantial advancements in the understanding of microcirculatory dysfunction in sepsis. In a seminal report published in 2002, De Backer and colleagues were the first to demonstrate direct evidence of impaired microcirculatory flow in the sublingual circulation of septic patients, using OPS imaging (De Backer et al., 2002). The authors observed a decrease in capillary density and in the proportion of perfused capillaries in patients with severe sepsis or septic shock, compared with healthy volunteers and other non-septic ICU patients. Furthermore these microvascular abnormalities were found to correlate strongly with disease severity and outcome: alterations were significantly more severe in non-survivors than survivors, and alterations in microvascular perfusion during the first day of ICU care were more strongly associated with outcome than cardiac output, venous oxygen saturation or arterial pressure. Interestingly, topical application of acetylcholine completely reversed the microvascular impairment, supporting the theory that compliant circulatory beds are actively shut down to maintain systemic haemodynamics during sepsis (Ince and Sinaasappel, 1999).

Since then there have been numerous reports of microvascular impairment in septic patients. In a small study of 49 septic shock patients, haemodynamic and systemic oxygenation variables were found to be equivalent between survivors and non-survivors, though microvascular perfusion was diminished to a greater extent in the latter (Sakr et al., 2004). The authors described an inverse relationship between microcirculatory perfusion and the development of multiple organ failure, and statistical analysis confirmed the prognostic value of small vessel perfusion 24 h after the onset of septic shock (71% sensitivity, 82% specificity). A 7.8% improvement in perfusion correlated with increased survival: ICU mortality was 19% compared with 71% in cases where intervention failed to improve perfusion by at least 7.8%.

These observations have been recapitulated in numerous clinical studies, using increasingly advanced technology, and microvascular alterations are now known to occur very early on in sepsis, often evident within a few hours of hospital admission (Trzeciak et al., 2008, Spanos et al., 2010). Using SDF imaging, Spanos and colleagues found that microvascular flow and the proportion of perfused vessels were significantly lower in patients with sepsis and severe sepsis than in healthy volunteers, and furthermore that the perfused vessel density appeared to correlate with severity (Spanos et al., 2010).

In the aforementioned study published by Davis and colleagues, microvascular reactivity in the index finger of severe sepsis patients was not significantly different between patients requiring vasopressors to maintain haemodynamic stability, and those who were not. In those receiving noradrenaline, there was no correlation between RH-PAT index and noradrenaline dose. Interestingly, RH-PAT index did not correlate with plasma markers of endothelial activation (e-selectin and intercellular adhesion molecule 1; ICAM-1), which were both significantly raised in septic patients compared with controls. Among severe sepsis patients, however, plasma lactate levels correlated inversely (albeit not significantly) with RH-PAT index. In this case, RH-PAT index did also correlate with arterial pressure. In septic patients there was no significant difference in RH-PAT index from baseline to days 2 to 4, though plasma L-arginine concentrations (which were markedly decreased at baseline) gradually increased over 4 days. Low baseline RH-PAT score significantly correlated with an increase in SOFA score over the first 4 days. The degree of impairment of baseline microvascular reactivity predicted subsequent deterioration in organ function (Davis et al., 2009).

Despite numerous reports demonstrating the prognostic value of measures of intestinal perfusion relative to systemic parameters (Gutierrez et al., 1992, Ivatury et al., 1996, Palizas et al., 2009), a 2009 trial in which resuscitation was targeted towards intestinal pH (pH_i) failed to show any survival benefit relative to cardiac index (CI) targets (Palizas et al., 2009). In this trial, however, correction of pH_i was only achieved in 7 out of 32 patients (22%) in the pH_i group – all of whom survived. Furthermore, patients in the pH_i group exhibited a higher SOFA score at the time of admission than patients in the CI group, and the timing of intervention (up to 48 h after ICU admission) varied greatly, such that patients in the former group may have experienced prolonged hypoperfusion and irreversible organ damage. This is in line with previous observations that pH_i -guided therapy is only effective in patients admitted with a normal pH_i , suggesting that the presence of mucosal acidosis at the time of admission is associated with irreversible organ dysfunction (Gutierrez et al., 1992).

A major potential factor in the failure of pH_i -oriented therapy regimens is the ineffectiveness of treatments aimed at normalising pH_i . This is reflected in the small proportion of patients in the pH_i group that actually achieved target pH_i values following intervention (22%), and may explain why mortality rates associated with severe sepsis and septic shock remain stubbornly high. Early intervention is undoubtedly key to limiting regional ischaemia, though as yet, no study has provided definitive evidence of a positive impact of therapies such as volume resuscitation and catecholamine administration on intestinal perfusion (Dubin et al., 2007, Gomersall et al., 2000).

The superiority of intestinal (or general microcirculatory) perfusion relative to global haemodynamic parameters as a predictor of outcome in sepsis has been established convincingly (De Backer et al., 2002, Gutierrez et al., 1992, Ivatury et al., 1996, Palizas et al., 2009, Sakr et al., 2004). Correspondingly (and even more so, given that no clinical intervention has been shown unequivocally to mitigate this perfusion failure), pre-clinical research should focus on the microcirculation (and particularly the splanchnic circulation) as an indicator of effectiveness in the development of novel anti-sepsis interventions. Given the prognostic sensitivity of markers of intestinal perfusion, pharmacological treatments that are shown to reverse dysfunction in this bed, are likely to have a significant clinical impact.

1.5 Poor research translatability

In the field of sepsis, pre-clinical research output has increased 5-fold over the past 20 years, and more than 100 multi-centre clinical trials of sepsis interventions have been conducted (Dyson and Singer, 2009). Despite this intense effort, only activated protein C (drotrecogin alpha; Xigris®) was approved for the treatment of sepsis, and this was later withdrawn from the market after failing to reduce mortality. It is clear that research translatability in the sepsis field is lacking considerably, though the reasons for this are not fully understood.

1.5.1 Animal models of sepsis

Over several decades, various pre-clinical models have been used to delineate the pathophysiological processes underlying sepsis-associated morbidity and mortality. They tend to be classified into one of three categories: intravenous infusion models, endotoxaemic models, or infectious models (Schultz and van der Poll, 2002). The latter predominantly comprise experimental pneumonia and experimental peritonitis models, as the lungs and the abdomen represent two of the most common sites of infection in septic patients (Martin et al., 2003). This thesis will focus on endotoxaemia and experimental peritonitis as pre-clinical models of sepsis.

1.5.1.1 Intravenous infusion of bacteria

Much pre-clinical sepsis research has involved intravenous infusion of large doses of bacteria, administered either continuously or as a bolus. This precipitates a rapid and profound drop in cardiovascular function, and animals tend to die within hours (Schultz and van der Poll, 2002). The hyperdynamic response observed in septic patients and described in section 4.4.1. can be reproduced in these animals by limiting the total dose of bacteria, prolonging infusion time, or by administering aggressive fluid resuscitation (Schultz and van der Poll, 2002); the resultant hyperdynamic state is also associated with improved survival (Fink and Heard, 1990).

Such studies are limited by a number of factors, however. Firstly, the bacteria used in these studies have tended to be serum-sensitive non-virulent strains that cannot multiply *in vivo*, and are thus rapidly killed upon administration (Schultz and van der Poll, 2002). Consequently, extremely large doses have been required to overwhelm host defences (Porat et al., 1992), such that physiological responses do not correlate well with the clinical syndrome; septic patients tend to have an infectious focus from which multiplying bacteria continuously disseminate over time (Schultz and van der Poll, 2002). Moreover, because mortality is relatively rapid in bacterial infusion models, the time for disease progression is limited, precluding comprehensive analysis of pathological mechanisms. The cytokine profile of such

models also varies across species and deviates greatly from the clinical situation: cytokine responses tend to be transient and of much greater magnitude in baboons, mice and rats than in septic humans, making it difficult to extrapolate results (Cross et al., 1993).

1.5.1.2 Endotoxaemic models

Systemic administration of the bacterial endotoxin lipopolysaccharide (LPS) initiates a physiological response that largely mirrors that observed in septic patients. Small sub-lethal doses of LPS induce a hyperdynamic cardiovascular response, while larger lethal doses precipitate a hypodynamic state (Schultz and van der Poll, 2002).

The LPS model confers several advantages over the administration of live bacteria. Firstly, LPS is stable, ensuring reproducible administration and easy storage. Secondly the quantity of LPS units infused can be easily titrated, and does not require bacterial culture and quantification. A major disadvantage of endotoxaemic models, as with intravenous infusion models, is the lack of infectious focus, however. Species differences are also observed: small mammals and baboons are relatively insensitive to LPS and require much larger doses than rats, sheep and chimpanzees (Schultz and van der Poll, 2002).

Nonetheless, a significant advantage of the LPS model is that it can be administered in low doses to healthy human volunteers in a controlled and reproducible manner, with minimal risk. Such experiments have contributed greatly to our understanding of cytokine networks and the initiation of inflammatory pathways (Schultz and van der Poll, 2002), and the haemodynamic effects of LPS are now well documented. Human subjects typically exhibit a hyperdynamic cardiovascular response, characterised by elevated cardiac index and heart rate, with decreased SVR and blood pressure (Suffredini et al., 1989).

1.5.1.3 Peritonitis models

The two most commonly used models of experimental peritonitis are cecal ligation and puncture (CLP), in which faecal peritonitis is induced surgically (Wichterman et al., 1980), and the fecal peritonitis model in which bacteria are suspended in a fibrin clot that is implanted into the abdominal cavity (Ahrenholz and Simmons, 1980). Intraperitoneal administration of virulent *Escherichia coli* (*E. coli*) has also been used to mimic peritonitis in pre-clinical models (Schultz and van der Poll, 2002). Animals typically develop a hyperdynamic response with increased cardiac output and decreased SVR, similar to human sepsis; thereafter they develop bacteraemia and cardiovascular collapse. There are several advantages to CLP over other methods of inducing peritonitis. Firstly, it is relatively simple, circumventing the need to grow and quantify bacteria; secondly it is clinically similar to problems such as perforated

appendicitis or diverticulitis – common causes of sepsis. Its polymicrobial nature is also more akin to the clinical situation; a large sample size can be used to compensate for problems of variability. The cytokine profile of experimental peritonitis models is similarly more representative of that observed in septic patients (Schultz and van der Poll, 2002).

1.5.1.4 Species selection and reproducibility

A broad range of animals has been used in pre-clinical sepsis research. While larger species facilitate high-volume resuscitation, small mammals (particularly mice) are often chosen for reasons of cost and practicality, the ease of genetic manipulation (facilitating mechanistic studies) and the homogeneity of genetic backgrounds, as well as the fact that they tend to be pathogen-free (Schultz and van der Poll, 2002). While genomic responses to inflammation may differ (Seok et al., 2013), physiological responses are well conserved between humans and rodents. In these animals, the primary endpoint has tended to be mortality, though with technological advancement, more complex assessment of haemodynamic function and immunological status has been rendered more feasible. Larger mammals, such as pigs, sheep, dogs and primates are often used in studies that require more invasive monitoring. The immunological similarities between primates and humans render this species more valuable in the assessment of cytokine responses.

A significant paradox of sepsis research centres on the control of confounding variables. While researchers must necessarily aim to limit the influence of confounding factors (such as age, gender, environment and genetic variation), in order to delineate the role of a particular protein or pathway in sepsis pathogenesis, the clinical situation is always heterogeneous: patients are exposed to different environmental factors and pharmacological stimuli, have massively different ages and a wide range of co-morbidities. Furthermore, in order to ensure consistency, the infectious or inflammatory insult must be titrated in pre-clinical studies, whereas septic patients will be exposed to vastly divergent levels of infectious material.

The difficulty of recapitulating an enormously complex and heterogeneous syndrome in a pre-clinical model is unquestionable. Nonetheless, animal research has led to the successful development of treatments for numerous complex diseases, including heart failure (Dell'Italia, 2011) and cancer (Hayes et al., 2014). That animal models can help to elucidate certain aspects of sepsis pathogenesis seems indubitable; that pre-clinical models currently do not confer sufficient clinical translatability, however, is equally certain, and significant refinement will be required to determine the probable clinical impact of novel interventions.

1.5.2 Notable cases of failed translatability

1.5.2.1 Blockade of NO production

The vasodilatory shock state elicited during sepsis progression was originally thought to result from excessive NO production by iNOS; pharmacological blockade of NO production was hypothesised to represent a viable treatment strategy, and correspondingly has been shown repeatedly to attenuate sepsis-associated hypotension in rats (Nava et al., 1992, Hwang and Yeh, 2003), mice (Ullrich et al., 2000) and sheep (Lorente et al., 1993b). Cardiac function and systemic haemodynamics were also found to be preserved in iNOS knockout mice injected with endotoxin, relative to wildtype controls (Ullrich et al., 2000), and genetic ablation of eNOS has similarly been associated with improved global haemodynamic performance in endotoxaemic mice (Connelly et al., 2005). Strikingly, however, infusion of methylene blue (a non-specific NOS inhibitor) was found to improve arterial pressure, stroke volume and left ventricular stroke work without altering outcome in septic patients (Kirov et al., 2001). Similarly, a large-scale multi-centre trial of N^G-monomethyl-L-arginine (L-NMMA) was aborted in phase III because of excessive mortality in the treatment group, despite a reduction in the requirement for vasopressor therapy and an increase in MAP (Lopez et al., 2004).

There are several possible explanations for the failure of these trials. Firstly, several recently published studies have found no net increase in NO synthesis in septic patients (Luiking et al., 2009, Kao et al., 2009, Villalpando et al., 2006). The formation of peroxynitrite radicals resulting from the reaction of NO with superoxide molecules produced in sites of excessive inflammation may prevent elevation of plasma NO levels. Another possibility is the reduced availability of L-arginine, the substrate for NOS and the precursor of NO: sepsis has been reported to be an 'arginine-deficient state', although reports of arginine levels have varied widely (Davis et al., 2009). An alternative explanation is an imbalance between iNOS and eNOS in the microvasculature (McGown and Brookes, 2007); a relative deficiency in eNOS-derived NO could compromise microvascular endothelial function.

It should be noted, however, that while the L-NMMA trial was aborted owing to excessively high mortality rates in the treatment group, post-hoc analysis revealed a slight improvement in mortality rates in patients receiving low doses of the drug (Lopez et al., 2004). The observation that MAP was raised in both high-dose and low-dose groups, however, suggests that survival may not have been directly linked to arterial pressure. Indeed a concomitant decrease in cardiac output was observed, though it is unclear whether this was representative of a physiological reflex to raised MAP or a direct effect of L-NMMA on the heart. Whatever the underlying mechanism, these studies emphasise the poor prognostic value of systemic

haemodynamic parameters. Given that endothelium-derived NO is essential for normal microvascular function, it is possible that excessive and non-selective blockade of NO production could severely compromise microcirculatory endothelial function in spite of improved systemic circulation, perhaps accounting for the increased mortality observed in patients treated with high doses of L-NMMA (Watson et al., 2004, Lopez et al., 2004). This is supported by the observation that sepsis-induced cardiac dysfunction is attenuated in female mice, who exhibit higher levels of cardiac eNOS phosphorylation during sepsis compared with male counterparts (Chen et al., 2014).

1.5.2.2 Activated protein C

Recombinant human activated protein C (APC, or drotrecogin alfa), an anticoagulant and anti-inflammatory agent, is the only pharmacological intervention to have been approved for the treatment of sepsis. APC inactivates factors Va and VIIIa in the clotting cascade, preventing the generation of thrombin, thereby disrupting feedback between the coagulation system and the inflammatory cascade (Matthay, 2001): inhibition of thrombin decreases inflammation by inhibiting platelet activation, neutrophil recruitment and mast-cell degranulation. APC has also been shown to have direct anti-inflammatory properties, including inhibition of cell adhesion and monocyte-induced cytokine production (Matthay, 2001).

Reduced levels of APC are frequently observed in patients with sepsis, and are associated with an increased risk of mortality (Fourrier et al., 1992, Lorente et al., 1993a, Boldt et al., 2000); consequently, it was hypothesised that restoration of APC could be beneficial in septic patients. Correspondingly, administration of APC to baboons injected with *E. coli* was found to prevent hypotension, limit coagulation and immune cell infiltration, attenuate liver histopathology and reduce mortality (Taylor et al., 1987). It was similarly found to attenuate leukocyte-induced lung injury in rats treated with LPS (Murakami et al., 1996).

Early studies in humans appeared promising: administration of drotrecogin alfa resulted in a 19.4% reduction in the relative risk of death, and an absolute risk reduction of 6.1% (Bernard et al., 2001). The occurrence of serious bleeding events (intracranial haemorrhage, a life-threatening bleeding episode, or a requirement for 3 or more units of blood) was significantly higher in the treatment group, however: 3.5%, compared with 2% on placebo. With open-label use after the trial, intracranial haemorrhage occurred in 13 out of 520 patients (Warren et al., 2002). Furthermore, subsequent clinical trials were unequivocally disappointing (Abraham et al., 2005, Nadel et al., 2007, Ranieri et al., 2012), and drotrecogin alfa (trade name Xigris®) was withdrawn from the market by its manufacturers Eli Lilly and Co. in 2011.

1.5.2.3 Others

Numerous other agents that have attenuated the global haemodynamic response to sepsis in pre-clinical studies have later failed in clinical trials. Aspirin and indomethacin were found to prevent hypotension in dogs (Fletcher and Ramwell, 1977) and cats (Parratt and Sturgess, 1975) injected with *E. coli*, but non-steroidal anti-inflammatory drugs (NSAIDs) have failed to confer a survival benefit in clinical trials (Bernard et al., 1997, Memis et al., 2004). A host of other interventions, including heparin, IL-1 receptor antagonists, statins and numerous anticoagulants and anti-inflammatory agents have shown improved outcome in animal models of sepsis and later failed in clinical trials (Fink, 2014), though an in-depth discussion of these studies is beyond the remit of this thesis.

1.5.3 Potential reasons for poor research translatability

Pre-clinical studies have historically been conducted on the assumption that sepsis is merely the result of exaggerated inflammation, as discussed in section 1.4. Numerous immunomodulatory drugs that have appeared to confer promising results in pre-clinical models of sepsis, however, have later failed in clinical trials (van der Poll, 2001). This is undoubtedly attributable – at least in part – to the failure of several pre-clinical sepsis models to recapitulate the cytokine profile of human sepsis, as discussed in section 1.5.1.2. Based on the results of bacterial infusion models, it was hypothesised that mortality was the result of a ‘cytokine storm’. Indeed, one pre-clinical study found that blockade of cytokines with methylprednisolone reduced mortality in primates infused with extremely high doses of *E. coli* (Hinshaw et al., 1981); other investigators similarly demonstrated that anti-TNF α therapy improved survival in baboons injected with bacteria (Tracey et al., 1987), suggesting that cytokine blockade could be beneficial in sepsis. However, clinical trials of steroids (Cronin et al., 1995) and anti-TNF α treatment (Bernard et al., 2014) have shown no effect in septic patients, and some studies have indicated that mortality is actually enhanced by anti-inflammatory treatment (Fisher et al., 1996, Bone et al., 1987).

Other potential reasons for the failure of clinical translatability are manifold. Pathogenesis in pre-clinical models (particularly intravenous infusion models) tends to be extremely acute, whereas the clinical profile is sub-acute or intermittent (Schultz and van der Poll, 2002). The absence of a septic focus undoubtedly also limits the clinical relevance of infusion and endotoxaemic models. Another potential factor, that has not received as much consideration as issues of species relevance, is that pre-clinical research may have focussed on sub-optimal endpoints. Crude mortality endpoints may not be particularly useful if animals are dying by different mechanisms than humans (particularly as mainstay clinical interventions are

infrequently used in pre-clinical models) and the limited relevance of immunological endpoints is underscored by the failure of immunomodulatory drugs in clinical trials, as well as the observation that the genomic responses to inflammation differ greatly between mice and humans (Seok et al., 2013).

Furthermore, although the physiological sequelae of sepsis are reproduced in animals, and systemic haemodynamics are routinely monitored in pre-clinical models of sepsis and septic shock, it is now clear that these parameters do not sufficiently predict the onset of organ failure (Sakr et al., 2004). Refinement of pre-clinical models to incorporate endpoints with greater clinical relevance may help to identify more efficacious drug targets – a critical goal of sepsis research, given the massive and growing healthcare burden associated with the syndrome. This is particularly urgent in the context of increasing antibiotic-resistance in sepsis-associated pathogens, since antibiotic therapy is currently the only causal line of treatment, with no specific anti-sepsis drugs currently available. Increased understanding of the vascular changes that underlie sepsis pathogenesis will facilitate the identification of novel drug targets.

1.6 A potential role for TRPV channels in sepsis

In addition to the refinement of pre-clinical models, there is a strong requirement for identification of novel drug targets in sepsis, given the dearth of specific anti-sepsis drugs currently available. There is now increasing evidence that several thermosensitive depolarising cation-permeable transient receptor potential (TRP) channels, most notably of the vanilloid (TRPV) subfamily, can influence physiological systems compromised in sepsis, and may represent potential therapeutic targets.

1.6.1 TRPV channels: an overview

The superfamily of mammalian TRP channels comprises six subfamilies established on the basis of sequence homology: TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPA (ankyrin), TRPP (polycystin), and TRPML (mucoliptin) (Earley and Brayden, 2010). All proteins are expressed as six-transmembrane domain subunits, which form functional homo- or heterotetramers, further diversified by complex transcriptional regulation and splicing, as well as considerable post-translational modification (Clapham, 2003).

TRP channels, originally identified as cellular sensors in *Drosophila* mutants (Swanson and Cosens, 1981), are gated by temperature, light, pressure and chemical stimuli (Earley and Brayden, 2010), and exhibit broad tissue distribution as well as highly diverse functions, ranging from audio-visual transmission to nociception and thermoregulation (Clapham, 2003). In recent years there has been growing interest in the vasoregulatory and inflammatory properties of TRP channels, and much research has focussed on the role of heat-sensitive TRPV1 in these settings, though other TRPV channels, most notably TRPV4, are similarly emerging as important regulators of vascular function, whose activity may be altered under inflammatory conditions.

1.6.2 Regulation of endothelial Ca^{2+} levels by TRP channels

Endothelium-dependent vasodilatation can occur through several different mechanisms, most of which are regulated by intracellular Ca^{2+} : NO production by eNOS is enhanced through a Ca^{2+} -calmodulin-dependent mechanism; Ca^{2+} -dependent activation of phospholipase A_2 (PLA_2) liberates arachidonic acid from cellular membranes, providing a substrate for the biosynthesis of vasodilatory molecules such as prostacyclin (PGI_2) and epoxyeicosatrienoic acids (EETs); and finally, elevated endothelial Ca^{2+} levels can directly activate Ca^{2+} -sensitive K^+ channels to initiate hyperpolarisation, which can spread electrically through myoendothelial junctions to hyperpolarise and relax underlying smooth muscle (Sullivan and Earley, 2013).

Coupling of these vasodilatory systems to vascular TRP channels could represent important mechanisms of vasoregulation. A common pathway for endothelial stimuli involves an increase in intracellular Ca^{2+} . Typically, there is an initial rapid Ca^{2+} transient representative of intracellular store release via IP_3 and/or ryanodine receptors, followed by a sustained Ca^{2+} elevation representative of Ca^{2+} influx from the extracellular space. For many years the molecular identity of the Ca^{2+} influx channels was unknown. The discovery of TRP channels, however, has provided significant new insight into the mechanisms of Ca^{2+} influx into endothelial and other cells. Indeed, TRP channels have been implicated in many endothelial functions, including control of vascular tone, regulation of vascular permeability, mechanosensation, vasoactive responses to oxidative stress, angiogenesis and vascular remodelling (Nilius et al., 2003, Yao and Garland, 2005). Indeed, members of all TRP families (with the exception of TRPML) have been identified in endothelial cells.

1.6.3 TRPV1 as a potential regulator of vascular function in sepsis

TRPV1, predominantly expressed in sensory nerves (Caterina et al., 1997), but also thought to be present in cardiovascular tissues (Baylie and Brayden, 2011), has been studied in the context of sepsis for a number of years. The importance of sensory nerve input in sepsis was initially inferred from two observations: first, the exacerbated inflammatory response to infection following sensory denervation of experimental animals (Bowden et al., 1996); and second, the raised plasma levels in septic patients of neuropeptides that are released downstream of TRPV1 activation (Joyce et al., 1990, Beer et al., 2002). More recently, an upregulation of TRPV1 and the neuropeptide calcitonin gene-related peptide (CGRP) has been demonstrated in the tongue and mesenteric perivascular nerves, respectively, of endotoxaemic rats (Orliac et al., 2007). This suggests that changes in TRPV1 and neuropeptide expression may play an important role in sepsis.

Indeed, a role for TRPV1 in mediating the fever response to endotoxin has been demonstrated both in sensory-denervated rats (Romanovsky, 2004), and TRPV1 knockout (KO) mice (Iida et al., 2005). TRPV1 has also been shown to protect against sepsis-induced cardiovascular dysfunction: Wang and colleagues (2008) demonstrated that recovery from endotoxin-induced hypotension and tachycardia in anaesthetised mice was strongly inhibited by the TRPV1 antagonist capsazepine (Wang et al., 2008a). Additionally, 24- and 48-hour survival of conscious LPS-treated rats was similarly improved following administration of a TRPV1 antagonist. Conversely, Ang and colleagues reported improved survival and attenuated organ dysfunction in septic mice pre-treated with capsazepine (Ang et al., 2010). A 5-fold higher dose of the drug was used in the latter study, however; since the selectivity of capsazepine is known

to be compromised at higher concentrations (Teng et al., 2004), the physiological effects recorded in the latter study may have been independent of TRPV1.

Further investigation in our laboratory has demonstrated that TRPV1 KO mice exhibit significantly enhanced hypotension compared to wildtype (WT) counterparts at 4 h post-LPS administration, and early signs of liver dysfunction, including significant liver plasma extravasation and raised levels of plasma aspartate aminotransferase, were apparent in LPS-treated TRPV1 KO but not WT mice (Clark et al., 2007). However, given that induction of cytokines, such as IL-6, only occurs approximately 6 h after the induction of endotoxaemia in mice, and haematological changes become pronounced after 24 h (Xiao et al., 2006), it seems likely that a 4-h end-point is too early to fully assess the functional effects of TRPV1 deletion in a murine model of sepsis. Furthermore, the possibility of developmental compensation for TRPV1 gene ablation cannot be ruled out. Selective and tissue-specific TRPV1 antagonism *in vivo* may be helpful in elucidating this further.

A more recent study, using the cecal ligation and puncture (CLP) model of sepsis (thought to better recapitulate the polymicrobial insult generally encountered in human patients) found that at 24 h post-CLP, TRPV1 KO mice exhibit enhanced hypotension compared to WT controls, and plasma markers of heart, liver, kidney and pancreas dysfunction were significantly raised in the former group (Fernandes et al., 2012). Decreased mononuclear cell integrity and enhanced peritoneal apoptosis were observed at the primary site of infection in the KO mice. Moreover, macrophages isolated from TRPV1 KO mice exhibited an impaired ability to phagocytose latex beads *in vitro*, following stimulation with LPS. This process in WT mice was found to be dependent on substance P – a neuropeptide released downstream of TRPV1 activation (Fernandes et al., 2012). These data suggest that deletion of the TRPV1 gene leads to impaired bacterial clearance in sepsis, accelerating the transition from local infection to a full systemic inflammatory response syndrome, and the onset of organ damage and failure.

Thus far, the longer-term effects of TRPV1 deletion on vascular function in sepsis have not yet been investigated. It is possible that, in addition to immune cell regulation, TRPV1 may also mediate vasoactive effects: given its ability to cause both vasoconstriction and vasodilatation, depending on its cellular localisation, TRPV1 may contribute to recovery from septic hypotension through the former mechanism, or the maintenance of organ perfusion through the latter. This may form a basis for understanding why septic TRPV1 KO mice appear to exhibit both exaggerated hypotension and accelerated organ dysfunction (Clark et al., 2007).

1.6.4 TRPV4 as a potential regulator of vascular function in sepsis

TRPV4, originally identified as an 'osmosensor' in the kidney by three different groups simultaneously (Strotmann et al., 2000, Liedtke et al., 2000, Wissenbach et al., 2000), is expressed in numerous tissues, including the heart and vasculature (Wissenbach et al., 2000). Its expression in both endothelial and smooth muscle cells is now well established (Baylie and Brayden, 2011), and in addition to hypotonicity, it is gated by various physical and chemical stimuli, including shear stress, arachidonic acid metabolites (particularly EETs) and endocannabinoids (Nilius et al., 2004) – all of which may be altered under inflammatory conditions. Recent reports linking excessive TRPV4 activation to lethal oedema formation, hypotension and endothelial failure, suggest it may play a key role in sepsis pathogenesis.

Hydrostatic stress is known to increase vascular permeability through stimulation of endothelial Ca^{2+} influx, Ca^{2+} -dependent activation of myosin light chain kinase (MLCK) and subsequent contraction of endothelial cells (Yin et al., 2008). As a mechanosensitive channel, numerous studies have implicated TRPV4 in the Ca^{2+} influx that occurs in response to raised hydrostatic pressure. Alvarez and colleagues showed that TRPV4 activation increases permeability in the lung microvasculature and preferentially causes barrier disruption in alveolar septal regions (Alvarez et al., 2006). Channel activation has also been shown to increase paracellular permeability with disruption of epithelial tight junctions (Reiter et al., 2006). The TRPV4 agonist 4 α -phorbol 12,13-didecanoate (4 α PDD) has been shown to cause pulmonary oedema in the absence of raised hydrostatic pressure, though it did not increase permeability further when pressure was already raised, suggesting convergent mechanisms that centre on TRPV4 (Yin et al., 2008). This is supported by the observation that pressure-induced Ca^{2+} influx and lung oedema are dramatically reduced in TRPV4 KO mice (Yin et al., 2008).

Furthermore, pressure-induced oedema formation in isolated lungs was attenuated by low- Ca^{2+} buffer, the TRPV4 blocker ruthenium red (RR), and ablation of the TRPV4 gene; gadolinium – a non-selective blocker of stretch-activated ion channels – did not enhance the inhibition further, suggesting that TRPV4 is exclusively responsible for the hyperpermeability response to raised hydrostatic pressure (Jian et al., 2008). Hyperpermeability and plasma extravasation are key features of sepsis pathogenesis, and it is possible that under inflammatory conditions, endogenous activators of TRPV4 could precipitate significant organ oedema in the absence of raised hydrostatic pressure.

TRPV4 has been implicated in both flow- and agonist-induced vasodilatation, in several different vascular beds (Kohler et al., 2006, Hartmannsgruber et al., 2007, Loot et al., 2008,

Zhang et al., 2009, Mendoza et al., 2010, Adapala et al., 2011, Zhang and Gutterman, 2011). Its coupling to Ca^{2+} -sensitive K^+ channels stimulates endothelial hyperpolarisation upon channel activation, which spreads to the underlying smooth muscle via myoendothelial gap junctions.

In 2008, Willette and colleagues demonstrated that systemic activation of TRPV4 caused profound hypotension and circulatory collapse; 0.3 mg/kg GSK1016790A (a selective TRPV4 agonist) administered intravenously caused death in 100% of WT mice (Willette et al., 2008). Mice heterozygous for TRPV4 exhibited an attenuated response to the agonist (the lethal dose was raised to 1 mg/kg), while no cardiovascular derangements were observed in TRPV4 KO mice. Continuous intravenous infusion of GSK1016790A also caused a profound drop in mean arterial pressure and systemic vascular resistance in dogs, who exhibited similar circulatory failure.

The authors suggested that the lethal effects of GSK1016790A were associated with endothelial barrier failure: massive haemorrhages were observed in the lung, kidney and intestine of animals treated with higher doses of the drug, though there was no evidence of microvascular injury in the heart, brain, liver or skeletal muscle, suggesting a heterogeneous response to TRPV4 activation. It is possible that the absence of epithelial TRPV4 expression in the latter tissues could account for this phenomenon: co-operativity between epithelial and endothelial channels (as encountered in the gut, kidney and lung) could facilitate barrier breakdown.

Administration of 10 nM GSK1016790A to cultured human umbilical vein endothelial cells (HUVECs) was found to cause rapid cell retraction and condensation, and subsequent detachment, supporting the hypothesis that circulatory collapse in response to systemic TRPV4 activation is associated with endothelial barrier failure (Willette et al., 2008).

In 2012, Sonkusare and colleagues investigated the molecular mechanisms of TRPV4-mediated vasoregulation in more detail. Using transgenic mice expressing a Ca^{2+} -biosensor exclusively in the endothelium, the authors demonstrated the existence of basal endothelial Ca^{2+} pulses in isolated mesenteric arteries, that dramatically increased in frequency in response to 10 nM GSK1016790A (Sonkusare et al., 2012). Administration of 10 nM GSK1016790A activated approximately three sparklets per cell, and this was sufficient to hyperpolarise the underlying smooth muscle and cause maximal relaxation of pressurised arteries. This dilatory response was unaffected by inhibitors of eNOS and cyclo-oxygenase, though charybdotoxin – an inhibitor of large-conductance Ca^{2+} -sensitive IK^+ channels – abolished dilatation in response to 3 nM GSK1016790A, and greatly reduced the dilation to 10 nM GSK1016790A.

Perforated patch clamping revealed an outward current in response to 10nM GSK1016790A that was substantially reduced by charybdotoxin and further reduced by subsequent addition of apamin – a blocker of small-conductance Ca^{2+} -sensitive SK^+ channels. In fact, 10nM GSK1016790A was found to increase the density of IK^+ and SK^+ currents to a similar degree to dialysis with 3 μM Ca^{2+} . Together, these results demonstrate a specific link between TRPV4 activation and endothelium-dependent hyperpolarisation, and may represent a molecular basis for the profound drop in blood pressure and systemic vascular resistance encountered in sepsis (Sonkusare et al., 2012).

Interestingly, the systemic and regional haemodynamic effects of TRPV4 activation are differentially regulated by NOS activity. Intravenous injection of GSK1016790A in rats was shown to cause a decrease in mean arterial pressure and systemic vascular resistance, while causing an acute and transient increase in pulmonary arterial pressure, followed by a drop. In the presence of L-NAME, however, GSK1016790A produced an enhanced decrease in systemic arterial pressure, and large dose-dependent increases in pulmonary artery pressure, followed by a small drop (Pankey et al., 2014). These observations suggest that under pathological conditions characterised by increased oxidative stress (which can inactivate NO), TRPV4-mediated Ca^{2+} entry may promote pulmonary vasoconstriction, increase hydrostatic pressure in the lung, precipitating oedema formation and limiting adequate oxygen perfusion.

1.7 Hypotheses and Aims

Given the dearth of effective clinical treatments for sepsis and septic shock, it is clear that more relevant pre-clinical models are essential to better understand disease progression and to test the effectiveness of novel pharmacological or genetic interventions. The hypotheses of this study were as follows:

- 1) Microvascular blood flow represents a more sensitive and clinically relevant endpoint in pre-clinical sepsis studies than global haemodynamics
- 2) Inflammation-induced upregulation of TRPV1 mediates protective effects through the ability of the receptor to regulate vascular function under inflammatory conditions
- 3) Blockade of TRPV4 will attenuate sepsis-associated endothelial failure and circulatory collapse

On the basis of these hypotheses, the aims of this study were as follows:

- 1) To investigate alterations in microvascular function following the onset of sepsis, and to relate these alterations to changes in global haemodynamic, biochemical and haematological parameters
- 2) To establish a robust model for assessing microvascular blood flow in sepsis, and to incorporate this model into a multi-parameter monitoring system, using gold standard monitoring techniques, to allow microvascular perfusion and global haemodynamics to be measured in the same animal *in vivo*, facilitating paired comparison and reducing the number of animals required
- 3) To use the aforementioned model to determine the impact of TRPV1 and TRPV4 modulation on sepsis-associated cardiovascular dysfunction in a murine model of sepsis
- 4) To establish *in vitro* models for assessing vascular TRPV1 and TRPV4 expression and activity under basal and inflammatory conditions, to elucidate the mechanisms by which these channels may regulate vascular function *in vivo*

CHAPTER 2 | GENERAL METHODS

2.1 Animals

Mice were maintained on a 12-h light/dark cycle (7am–7pm, and 7pm–7am, respectively) and given access to food (normal chow) and water *ad libitum*. All experiments were carried out under license (PPL 70/7049) in accordance with the Animals (Scientific Procedures) Act, 1986, and UK Home Office regulations. In each experiment, animals were age- and gender-matched, and the researcher was blinded to genotype throughout the practical and analytical stages of experimentation. All treatments were administered in a randomised manner.

2.1.1 Origin of genetically altered mice

2.1.1.1 TRPV1 knockout mice

C57/BL6 mice, either genetically unaltered (WT) or with a non-functional TRPV1 receptor gene (TRPV1 KO), were bred in house from mice generously donated to Professor Sue Brain by Merck, Sharp & Dohme (Harlow, UK). TRPV1 KO mice were originally generated by Merck, Sharp & Dohme via replacement with a neomycin cassette of an exon on chromosome 11B3 encoding part of the fifth and the entire sixth transmembrane domain of TRPV1, including the interconnecting pore-forming loop, as shown in **Figure 4** (Caterina et al., 2000).

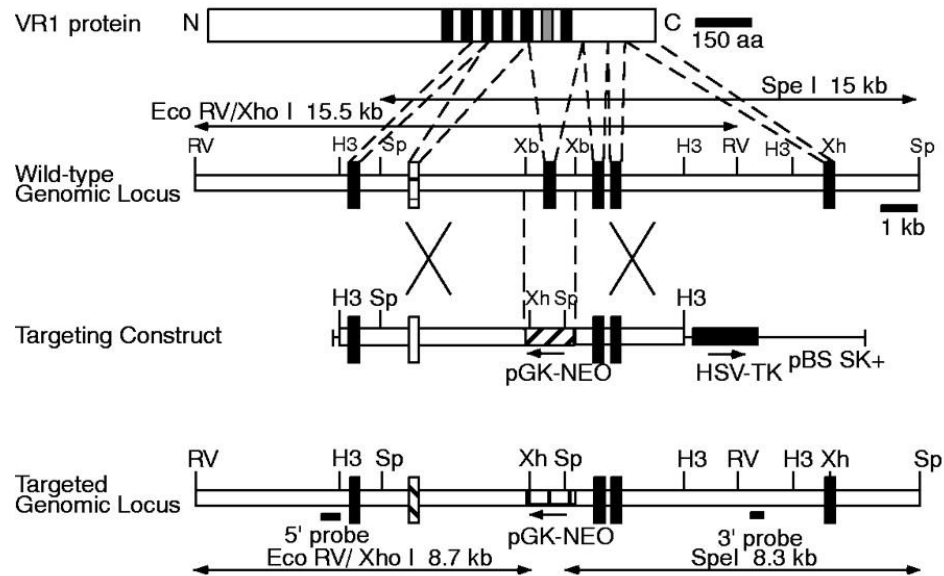


Figure 4. Strategy for TRPV1 (previously VR1) gene disruption, as conducted by Caterina et al. (2000). Black and grey vertical bars denote transmembrane and pore-loop domains of TRPV1, respectively. Exons encoding the carboxy-terminal portion of TRPV1 are indicated by vertical bars on the genomic maps. Part of the 5th and the entire 6th transmembrane domain of TRPV1 are replaced with a neomycin cassette. RV, Eco RV; H3, Hind III; Xb, Xba I; Xh, Xho I; Sp, Spe I; aa, amino acid. Figure adapted from Impaired Nociception and Pain Sensation in Mice Lacking the Capsaicin Receptor, Caterina et al. (2000). Science (Caterina et al., 2000).

TRPV1 KO mice show no aversion to drinking water supplemented with 100 μ M capsaicin, and exhibit a reduced sensitivity to thermal pain and reduced inflammatory hyperalgesia (Caterina et al., 2000, Davis et al., 2000). They are otherwise phenotypically indistinguishable from WT littermates, with regard to general appearance, behaviour, blood pressure, gross anatomy and life span (Caterina et al., 2000, Davis et al., 2000).

2.1.1.2 TRPV4 knockout mice

C57/BL6 mice with a non-functional TRPV4 receptor gene (TRPV4 KO) were re-derived from frozen embryos generously donated by Dr Andy Grant, Wolfson Centre for Age-Related Disease, King's College London, and were originally derived from a colony generated by Liedtke & Friedman in Germany (Liedtke and Friedman, 2003). Disruption of the TRPV4 gene was achieved through *cre-lox*-mediated excision of exon 12 of the TRPV4 gene, encoding transmembrane domains 5 and 6 and the putative pore-forming loop, and subsequent insertion of a neomycin cassette.

TRPV4 KO mice are viable and fertile and are indistinguishable from WT littermates on a macroscopic level, although KO mice do exhibit altered osmotic regulation and mechanical sensitivity (Liedtke and Friedman, 2003).

2.1.2 Breeding

A heterozygous breeding strategy was employed to generate WT, KO and heterozygous littermates. TRPV1 KO mice were born in the expected Mendelian ratios of genotype and gender, though TRPV4 KO mice were not: only 5 male TRPV4 KOs were born following multiple rounds of breeding by 8 heterozygous pairs.

Mice were weaned at 3 weeks of age and were split into cages according to gender. WT and KO littermates were housed together until experimentation. Following the induction of sepsis, mice were all singly-housed.

2.1.3 Genotyping

2-mm ear punches were taken from mice at 3 weeks of age, both to individually mark mice and procure a tissue sample. DNA was extracted by digesting tissue samples in DirectPCR lysis reagent (Viagen, 102-T) with proteinase K at 55°C overnight. Samples were then heated to 85°C for 45 min, centrifuged, and either used directly or stored at -20°C. Following *in vivo* experiments, post-mortem genotypes were confirmed through similar methods.

For PCR amplification, 2 µl genomic DNA was added to 18 µl ReddyMix™ PCR Master Mix (Thermoscientific, AB-0575/LD/A) containing specific primers (500nM each). PCR products were resolved on a 2% agarose gel at 80 V for 50 min.

2.1.3.1 TRPV1 knockout mice

PCR amplification of WT and mutant TRPV1 was performed on genomic DNA samples using the following primers:

- a. TRPV1 WT forward: 5'-CCT GCT CAA CAT GCT CAT TG-3'
- b. TRPV1 KO forward: 5'-TGG ATG TGG AAT GTG TGC GAG-3'
- c. TRPV1 common reverse: 5'-TCC TCA TGC ACT TCA GGA AA-3'

The following thermocycling conditions were used to amplify PCR products:

	Temp (°C)	Time
Initial denaturation	94	5 min
Amplification (x40 cycles)	94	30 s
	62	30 s
	72	1 min
Final extension	72	5 min

Table 2. PCR thermocycling conditions for amplification of TRPV1 WT and KO DNA

The TRPV1 WT forward primer (binding exclusively to wildtype DNA) and TRPV1 common reverse primer (binding downstream of the neomycin cassette, and so common to both genotypes) produced an amplicon of 984 bp. The TRPV1 KO forward primer (binding in the neomycin cassette) and TRPV1 common reverse primer produced an amplicon of 450 bp. Both bands were observed in samples from heterozygous mice (**Fig. 5**).

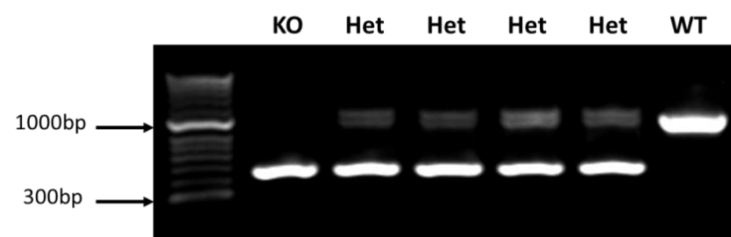


Figure 5. Agarose gel visualised under UV illumination to show products of PCR amplification from TRPV1 KO (450 bp), WT (984 bp) and heterozygous (Het) mice (450 bp and 984 bp).

2.1.4 TRPV4 knockout mice

TRPV4 WT, KO and heterozygous mice were distinguished by PCR amplification of two distinct transcripts: a long transcript of 1.1 kb, present in mutant TRPV4 DNA, and a short transcript of 251 bp from within exon 12 of WT DNA. The following primers and thermocycling conditions were used for amplification of the long transcript:

- a. TRPV4 long forward: 5'-CAT GAA ATC TGA CCT CTT GTC CCC-3'
- b. TRPV4 long reverse: 5'-TTG TGT ACT GTC TGC ACA CCA GGC-3'

	Temp (°C)	Time
Initial denaturation	94	5 min
Amplification (x40 cycles)	94	30 s
	60	1 min
	72	2 min 30 s
Final extension	72	10 min

Table 3. PCR thermocycling conditions for amplification of TRPV4 KO DNA

Amplification of the short transcript was achieved with the following primers and thermocycling conditions:

- a. TRPV4 short forward: 5'- AGG GCG ATA AGC ATG TTC AAC AGG-3'
- b. TRPV4 short reverse: 5'- TGC ACC AAC ATG AAG GTC TGT GAC G-3'

	Temp (°C)	Time
Initial denaturation	94	5 min
Amplification (x40 cycles)	94	30 s
	60	1 min
	72	1 min
Final extension	72	5 min

Table 4. PCR thermocycling conditions for amplification of TRPV4 WT DNA

In WT samples, short transcript primers produced a band of 251 bp, and no long transcript was produced. KO samples produced a band of 1.1 kb with the long transcript primers only. Both bands were observed in samples from heterozygous mice (**Fig. 6**). Products were visualised following electrophoretic separation on a 1% agarose gel (80 V, 30–60 min), and ethidium bromide staining under UV illumination.

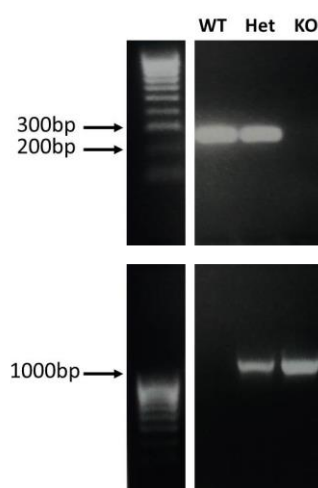


Figure 6. Agarose gel visualised under UV illumination to show products of PCR amplification of TRPV4 WT, KO and Het DNA. with short transcript primers were loaded into the top wells; products of amplification with long transcript primers into the lower wells. A single band of 1.1 kb indicates TRPV4 KO DNA; a single band of 251 bp indicates TRPV4 WT DNA. Heterozygous (Het) samples produced both bands.

2.2 Anaesthesia

Anaesthesia was induced with inhaled 3% isoflurane (Abbott Laboratories, UK), and was maintained under 1.8 – 2.2% isoflurane, delivered by air pump. A deep level of anaesthesia was confirmed by the absence of a paw-pinch reflex. Intramuscular buprenorphine hydrochloride (Vetergesic; Alstoe Animal Health, UK) and subcutaneous saline resuscitation were administered in all recovery procedures, and cervical dislocation or terminal venous bleed were carried out under isoflurane-induced anaesthesia for terminal procedures. All procedures were carried out using a homeothermically-controlled heating mat coupled to a rectal temperature probe (Harvard Instruments, UK). Viscotears liquid gel (Novartis, UK) was used to protect eyes during prolonged anaesthesia (>1 min).

2.3 Sepsis Models

Two models of sepsis were employed for this study: intravenous endotoxaemia and polymicrobial peritonitis. Both will be referred to as 'sepsis' throughout this thesis.

2.3.1.1 Lipopolysaccharide-induced endotoxaemia

Endotoxaemia was induced by intravenous injection of lipopolysaccharide (LPS; 12.5mg/kg) from *Salmonella typhimurium* (Sigma, L7261). Injections of approximately 100 µl (pre-warmed to 37°C) were administered into the tail vein (using a 29 G needle and insulin syringe) under inhaled isoflurane anaesthesia. A heating mat set to 37°C was used in order to dilate the tail vein for improved accessibility. Buprenorphine hydrochloride (15 µg/kg, i.m.) was administered into the two hind-limbs to provide post-operative analgesia and 0.9% (w/v) saline was administered subcutaneously to provide fluid resuscitation. Mice were placed in a recovery chamber at 27°C for up to 24 h and were monitored frequently. An arbitrary severity score of 1–5 (based on mobility, facial expression, piloerection and aversion to touch) was employed to assess animal welfare. Any mouse reaching a score of 5 was immediately terminated.

2.3.1.2 Cecal ligation and puncture

Polymicrobial peritonitis was induced by cecal ligation and puncture, as described previously (Rittirsch et al., 2009). Mice weighing 25–30 g were anaesthetised under inhaled isoflurane delivered by air pump, and placed in the supine position on a homeothermically-controlled heating pad. Buprenorphine hydrochloride (15 µg/kg, i.m.) was administered into the two hind-limbs to provide post-operative analgesia. The abdominal region was shaved and disinfected with chlorhexidine, and sterile surgical drapes were used to isolate a small abdominal region. Following midline laparotomy (1 cm), the cecum was exteriorised onto moist sterile gauze and a silk ligature was placed distal to the ileocecal valve, and tightened (bowl continuity was preserved). A single through-and-through puncture using a 19 G needle was then made in the ligated portion of the cecum, and gentle pressure was used to express a small droplet of faecal matter through each puncture site. The cecum was then returned to the abdominal cavity, which was closed using 5.0 Vicryl sutures. A step-by-step summary of the procedure is shown in **Figure 7**. For control sham operations, the ligature was not tightened, and no puncture was made. Saline (0.9% w/v) or Hartmann's fluid was administered subcutaneously to provide fluid resuscitation, and animals were allowed to recover at 27°C for up to 24 h with frequent monitoring.

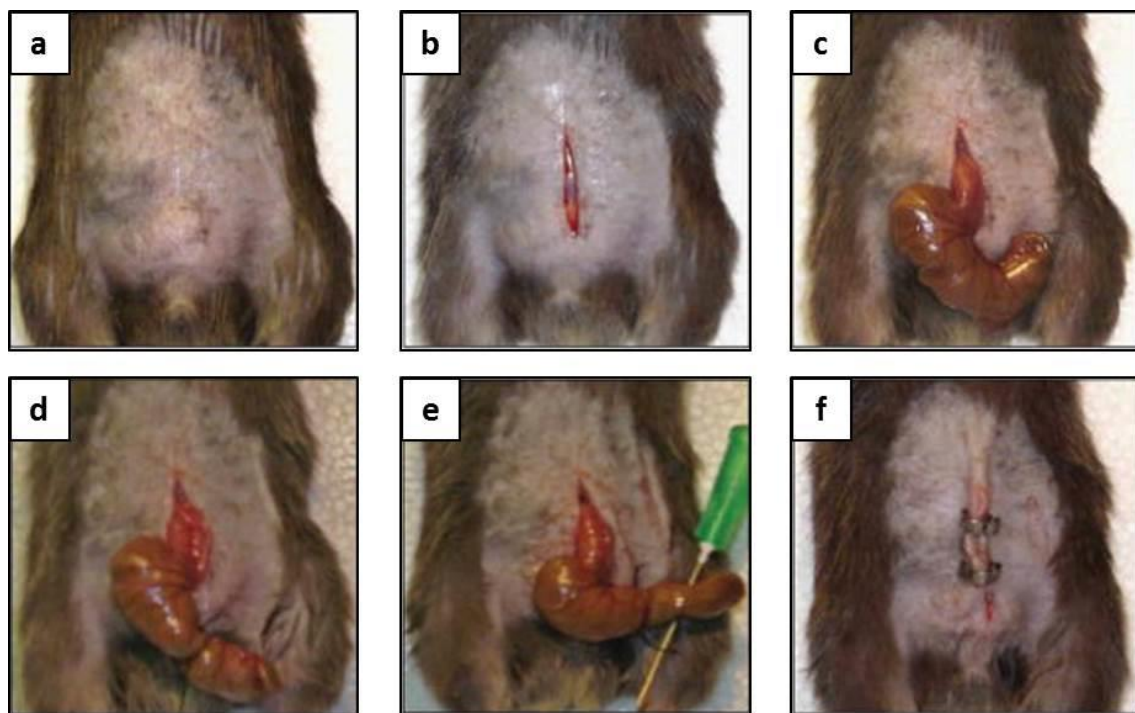


Figure 7. Critical steps in the CLP procedure developed by Rittirsch et al. (a) The abdominal area was shaved and disinfected with chlorhexidine. (b) A 1-cm midline incision was made with sterile scissors. (c) The cecum was exteriorised (onto moist sterile gauze in the current study). (d) The terminal third of the cecum was ligated with a silk ligature. (e) A single through-and-through puncture was made using a 19 G needle, and gentle pressure was applied with blunt forceps to express a small droplet of faecal matter through each puncture site. (f) The cecum was returned to the abdominal cavity and the incision closed with 5.0 Vicryl sutures (metal clips were not used in the current study). Figure adapted from Immunodesign of experimental sepsis by cecal ligation and puncture, Rittirsch et al. (2009). *Nature Protocols* (Rittirsch et al., 2009).

2.4 Haemodynamic assessment by radiotelemetry

PA-C10 telemetry probes (Data Sciences International; DSI, USA) were implanted into 10–12-week-old mice weighing 25–30g, under inhaled isoflurane anaesthesia using aseptic technique. Mice were placed in the supine position on a homeothermically-controlled heating mat, and the thorax was shaved and disinfected with chlorhexidine. Buprenorphine hydrochloride (15 µg/kg, i.m.) was administered into the two hind-limbs to provide post-operative analgesia. A small thoracic incision was made and the carotid artery was isolated by gentle blunt dissection. Telemeter catheters were placed in the left carotid artery and secured with three silk sutures. The body of the telemetry probe was placed in a subcutaneous pocket equidistant between the fore- and hind-limb, and the incision was closed using a 5.0 Vicryl suture. Mice were then placed in a recovery cabinet at 27°C for 4 h, before being moved to a holding room, where they were allowed to recover for at least 10 days before the start of haemodynamic monitoring. The viability of each haemodynamic trace was checked, and mice with significant dampening or loss of signal were excluded from analysis, as shown in **Figure 8**. All implantation, monitoring and data analysis was conducted in a blinded fashion, and assignment of telemetry probes was randomized.

PA-C10 telemetry probes allow continuous and remote monitoring of blood pressure, heart rate and locomotor activity in freely moving conscious animals. Data were acquired continuously at 500 Hz using standard acquisition software (DSI, USA). Baseline recording was carried out over weekends in order to minimise disruption.

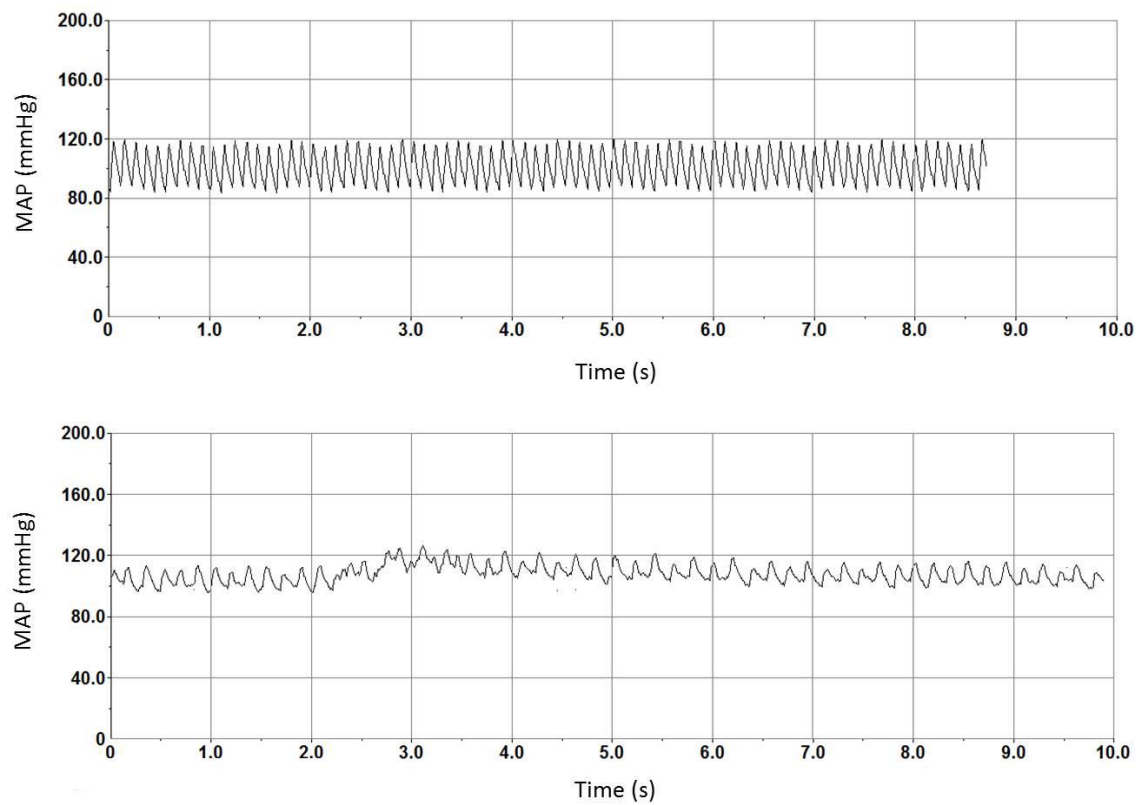


Figure 8. Representative traces of mean arterial pressure (MAP). Upper panel: acceptable pressure trace, indicating accurate positioning of pressure catheter. Lower panel: disrupted signal due to movement of the pressure catheter, leading to exclusion of this mouse from analysis.

2.5 Measurement of cardiac function by echocardiography

Echocardiographic monitoring of cardiac function was performed *in vivo* under isoflurane anaesthesia by Dr Anna Starr. Mice were placed on a homeothermically-controlled table and limb leads were attached for electrocardiogram gating. Images were acquired in the left lateral decubitus position with a 30 MHz linear probe (Visualsonic Vevo 770™, 30 MHz linear signal transducer). Two-dimensional images in parasternal long- and short-axis projections were recorded with guided M-mode recordings at the midventricular level in both views. Interventricular, septal and left ventricular posterior wall dimensions were taken in diastole and systole, in addition to left ventricular internal dimensions.

2.6 Measurement of microcirculatory perfusion

A detailed description of microcirculatory perfusion monitoring, including images of the set-up used, is provided in **Chapter 3**. Briefly, male mice aged 12–16 weeks were anaesthetised by inhaled isoflurane (2%, delivered by continuous air pump) and were placed in the supine position on a homeothermically-controlled heating mat, coupled to a rectal temperature probe. A small abdominal incision was made through which a portion of the small intestine was exteriorised onto a parafilm-coating heating mat. The intestine was pinned out through the gut wall to expose the mesenteric vasculature, and gently sprayed with saline pre-warmed to 37°C. Care was taken to avoid stretching or puncturing any blood vessels, and mice with significant bleeding were immediately terminated and excluded from analysis (approximately 7% throughout the course of this study). An additional heating mat was placed over the body of the anaesthetised mouse, and core temperature was continuously monitored.

Mesenteric blood flow was measured using a full-field laser perfusion imaging system (moorFLPI, Moor Instruments, UK) and moorFLPI Measurement software (Moor Instruments, UK). The following acquisition settings were used:

- High resolution capture (100 frames; 1 second/frame)
- Exposure time 20 ms
- Automatic gain
- Flux palette 0 – 5000 units
- Background threshold 60 flux units

After baseline recording, vasoactive drugs were administered topically or systemically. Data were analysed using moorFLPI Review software (V 4.0; Moor Instruments, UK) and GraphPad Prism (V 5.0; GraphPad Software Inc, USA). Vessels within the field of view were designated as 1st, 2nd or 3rd order branches and regions of interest, in which flux over time was measured,

were defined in each visible vessel (**Fig. 9**). Results are expressed as mean area and the curve (AUC) over time \pm SEM.

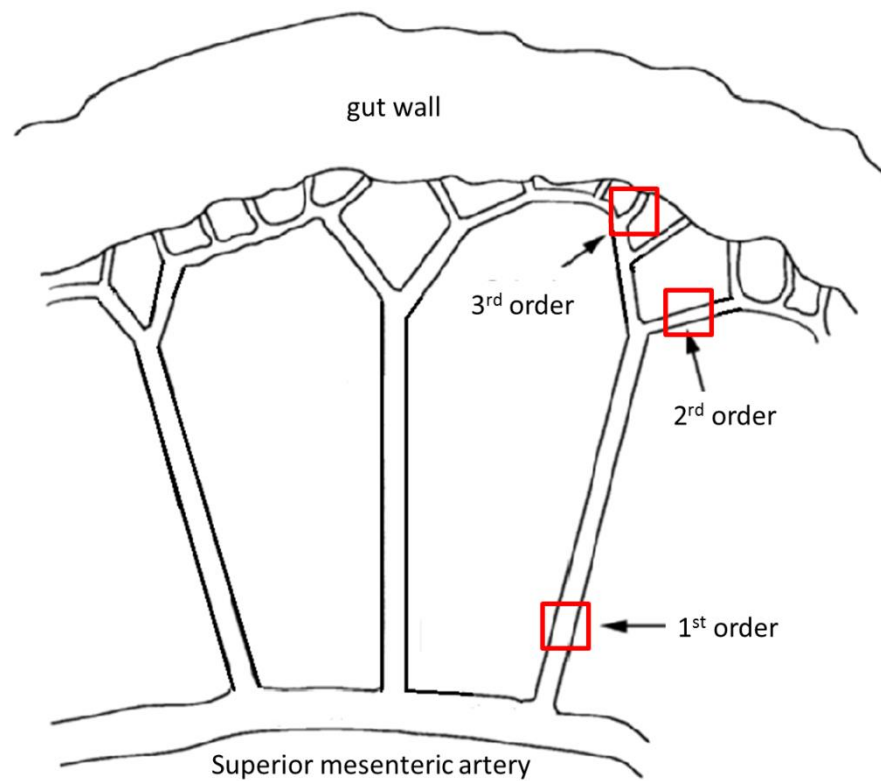


Figure 9. Schematic representation of the mesenteric vasculature. Vessels were designated as 1st, 2nd or 3rd order descendants of the superior mesenteric artery, and regions of interest were drawn as indicated by red squares, in order to measure blood flow in individual vessels.

2.7 Ex vivo analysis and tissue collection

2.7.1 Termination

Animals were terminated by exsanguination via the vena cava, followed by cervical dislocation. Venous blood samples were analysed immediately following venupuncture. Samples were then centrifuged (1200 rpm, 4 min), and the separated plasma fraction was snap-frozen in liquid nitrogen.

2.7.2 Analysis of blood biochemistry by iSTAT blood-gas analyser

Blood gases, biochemistry and composition were measured from 100 µl venous blood samples using a handheld iSTAT Point-of-Care analyser (Abbott Laboratories, USA) and EC8+ cartridges (Quality Clinical Reagents, UK) within 1 min of collection from the inferior vena cava (IVC).

2.7.3 Tissue collection

2.7.3.1 Assessment of oedema formation

Tissues were excised immediately after terminal IVC bleed and cervical dislocation and were weighed on a microbalance, before placement in a drying oven at 50°C, until a constant dry weight was reached (approximately 3 days). Dry organs were re-weighed, and ratios of wet weight:dry weight were calculated to determine the level of tissue oedema.

2.7.3.2 Tissue preparation for Western blot analysis

Following cervical dislocation and puncture of the inferior vena cava, animals were perfused with ice-cold saline injected into the left ventricle of the heart. Tissues were then excised, snap-frozen in liquid nitrogen and stored at -80°C. Frozen samples were lysed by mechanical homogenisation with stainless steel beads (30 Hz, 2-4 min) in RIPA buffer [20 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1 mM Na₂EDTA; 2.5 mM sodium pyrophosphate; 1 mM β-glycerophosphate; 1 mM Na₃VO₄; 1% NP-40; 1% sodium deoxycholate; 1 µg/ml leupeptin; Complete EDTA-free protease inhibitor cocktail (1 tablet/ 10 ml)]. Samples were then centrifuged and the pellet discarded. Supernatants were mixed with 4x Laemmli buffer [Final concentration: 2% (w/v) SDS; 10% (w/v) glycerol; 30mM Tris-Cl (pH 6.8); 0.01% (w/v) bromophenol blue; 5% (w/v) β-mercaptoethanol] and boiled at 95°C for 5 min prior to use.

2.8 SDS-PAGE and Western Immunoblotting

Protein concentration was determined by bicinchoninic acid (BCA) assay (Thermoscientific, 23227), using a bovine serum albumin (BSA) standard curve range diluted in RIPA buffer, as per kit instructions. 30 µg protein was added to each well of a 6% SDS-polyacrylamide gel and size-separated by electrophoresis at 200 V for 60 min. Proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane (GE Healthcare, RPN303F) by semi-dry transfer at 45 mA/membrane for 2 h and non-specific binding sites were blocked in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T) and 10% non-fat milk. Membranes were incubated with primary antibody on an orbital shaker at 4°C overnight, washed (4 x 10 min) with TBS-T, and then incubated with horse radish peroxidase (HRP)-conjugated secondary antibody at RT for 1 h. Immunoblots were developed using enhanced chemiluminescence (ECL; Thermoscientific, 32209) and a dark room imaging system, and signals were captured on autoradiographic film (GE Healthcare, 95017). Digital representations were generated by optical scanning. Only linear contrast adjustments were used, and were applied to the image as a whole. Details of the antibodies used are found in **Table 5**.

Antibody	Manufacturer	Catalogue No	Species	Vehicle (in PBS)	Working dilution
<i>Primary antibodies</i>					
Anti-TRPV1	Alomone Labs	ACC-030	Rabbit	1% BSA, 0.05% NaN ₃ in PBS	1 : 200
Anti-TRPV1	Abcam	ab45759	Mouse	1% BSA, 0.05% NaN ₃ in PBS	1 : 200
Anti-TRPV1	Abcam	ab31895	Rabbit	1% BSA, 0.05% NaN ₃ in PBS	1 : 200
Anti-TRPV1	Biorbyt	orb13755	Rabbit	1% BSA, 0.05% NaN ₃ in PBS	1 : 100
Anti-TRPV1	Biorbyt	orb32072	Rabbit	1% BSA, 0.05% NaN ₃ in PBS	1 : 100
Anti-β-actin	Sigma			1% non-fat milk in TBS-T	1 : 2000
<i>Secondary antibodies</i>					
Anti-rabbit	Cell Signaling	#7074	Goat	1 % non-fat milk in TBST-T	1 : 2000
Anti-mouse	Sigma	A4416	Goat	1% non-fat milk in TBST-T	1 : 5000

Table 5. List of antibodies used in Western blot analysis

2.9 Cell isolation and culture

2.9.1 Bovine aortic endothelial cells

Bovine aortic endothelial cells (bAEC) were isolated from fresh bovine aortae obtained from ABP Beef abattoir (Guildford, UK). Adherent fatty tissue was removed from the vessel, and the aorta was cut lengthways between intercostal arteries. Endothelial cells from the luminal surface of the vessel were scraped using the blunt side of a scalpel into pre-warmed Dulbecco's modified Eagle's medium (DMEM; PAA, E15-009) containing 0.2 mg/ml collagenase type 1A (Sigma, C9891), and incubated at 37°C for 20 min. The digest was then centrifuged (1200 rpm, 5 min) and the pellet resuspended in DMEM supplemented with 10% foetal bovine serum (FBS), 1% penicillin/streptomycin and 2 mM L-glutamine (complete DMEM) and seeded into a T75 (75 cm²) flask pre-coated with 0.5% gelatin (Sigma, G1393). Cells were allowed to adhere at 37°C in a 5% CO₂ humidified atmosphere for 4 h, before the culture medium was changed, and replaced every 48 h until cells reached confluency (approximately 1 week). The purity of endothelial cell cultures was determined on the basis of their characteristic cobblestone morphology.

2.9.2 Murine pulmonary endothelial cells

TRPV1 WT and KO mice were terminated by cervical dislocation under isofluorane-induced anaesthesia. Blood was flushed out of vessels by gentle cardiac perfusion with ice-cold phosphate-buffered saline (PBS), and lungs were harvested into ice-cold complete DMEM. Lungs were minced finely with a sterile scalpel blade and scissors, and digested at 37°C for 60 min with gentle agitation in 10 ml complete DMEM containing 0.18 U/ml Liberase Blendzyme 3TM (Roche, 05401119001) and 0.1 mg/ml DNase I (Roche, 11284932001). The digest was gently sheared through a 5 ml serological pipette and passed through a 70 µm filter. The filtered digest was centrifuged at 1200 rpm for 5 min at RT, and the pellet resuspended in PBS containing 0.5 % BSA and 2 mM EDTA (MACS[®] wash buffer). The resulting suspension was passed through a 40 µm strainer and centrifuged at 1200 rpm for a further 5 min at RT. The pellet was resuspended in 500 µl MACS[®] wash buffer and incubated with 1 µg/ml CD31 antibody (BD Pharmingen, 553369) for 15 min at 4°C. Following centrifugation (1200 rpm, 5 min, RT) the pellet was resuspended in 500 µl MACS[®] wash buffer and incubated with MACS[®] goat anti-rat IgG Microbeads (Miltenyi Biotec, 130-048-501) for 15 min at 4°C. The suspension was made up to 1 ml with MACS[®] wash buffer and centrifuged at 1200 rpm for 5 min at RT. The pellet was resuspended in 500 µl MACS[®] wash buffer and added to a MACS[®] separation column placed in a MACS[®] magnetic separator. The column was rinsed several times with MACS[®] wash buffer, and the flow-through discarded, before bound cells were eluted by

plunger. After a final centrifugation step (1200 rpm, 5 min, RT) cells were resuspended in endothelial basal medium (EBM) supplemented with endothelial growth supplement cocktail (EGM-2 MV BulletKit; Lonza, CC-3202), and plated into 6-well plates pre-coated with 0.5% gelatin. Experimentation was performed once cells had reached at least 80% confluency (approximately 7 days).

2.9.3 Murine aortic smooth muscle cells

TRPV1 WT and KO mice were terminated by cervical dislocation under isofluorane-induced anaesthesia. Blood was flushed out of vessels by cardiac perfusion with ice-cold phosphate buffered-saline (PBS) and the aorta was isolated, cleaned of surrounding fat tissue and cut open lengthways. Endothelial and adventitial layers were removed by scraping both sides of the vessel with a sterile scalpel blade, and the aorta was then cut into small pieces and digested for 60 min at 37°C with gentle agitation in DMEM containing 1.5 mg/ml collagenase type 2 (Worthington Labs, LS004196). The digest was passed through a 70 µm strainer and centrifuged at 1200 rpm for 5 min at RT. Cells were washed once more in DMEM and re-centrifuged, and the pellet was resuspended in DMEM containing 20% FBS. After 3 days, FBS was reduced to 10% and the cells were used after 7 days.

2.9.4 Human umbilical vein endothelial cells

Primary human umbilical vein endothelial cells (HUVEC) were kindly donated by Dr Bijal Patel (King's College London, UK). Cells were isolated by collagenase digest similar to the isolation of bAEC, described above, and were used at passage 4.

2.9.5 Murine bone marrow-derived macrophages

Bone marrow-derived macrophages (BMDM) were isolated from a colony of TRPV4 WT and KO mice at the Wolfson Centre for Age-Related Diseases (King's College London). Mice were terminally anaesthetised with sodium pentobarbital (100 mg/kg, i.p.) and femur and tibia bones were removed and separated following gentle dissection of overlying skin and muscle. Isolated bones were cleaned with an ethanol swab and transferred to a dish of DMEM. The ends of all four bones (two from each hind-limb) were cut with sterile scissors. A 10-ml syringe with a 25-G needle containing ice-cold $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS was inserted into the bone marrow cavity, and the bone marrow flushed out with 2–5 mL PBS. Cells were centrifuged at 2000 rpm for 7 min at RT, and the pellet resuspended in DMEM containing macrophage colony-stimulating factor (25 ng/mL of 500 U/mL stock; Sigma-Aldrich; DMEM-MCSF). The suspension was passed through a 40-µm filter and the flow-through made up to 25 mL with DMEM-MCSF supplemented with 10% foetal bovine serum (FBS), 1% penicillin/streptomycin and 2 mM L-

glutamine (complete DMEM-MCSF). Cells were then plated in a 15-cm dish and incubated at 37°C in a 5% CO₂ humidified atmosphere. After 5–7 days, culture supernatants were gently aspirated, and cells were gently washed with pre-warmed PBS. Following 5-min incubated at 37°C in PBS, cells were gently scraped off the plastic dish, centrifuged at 1500 rpm for 7 min at RT, and resuspended in complete DMEM-MCSF and plated onto glass coverslips (200,000 cells/coverslip) for imaging.

2.9.6 Immortalised cell lines

In addition to primary cultures, a number of immortalised cell lines were used: mouse skin endothelioma cells (sEnd1) were originally obtained from Dr E.F. Wagner (European Molecular Biology Laboratory, Heidelberg, Germany). Human embryonic kidney (HEK) cells and HEK cells stably expressing rat TRPV1 (HEK-TRPV1) were kindly supplied by Dr A. Grant (King's College London, UK).

2.9.7 General cell culture

All cells were maintained in complete DMEM or EBM at 37°C in a 5% CO₂ humidified incubator. Subculture was performed on confluent cells by washing twice with pre-warmed PBS, and incubating at 37°C with trypsin EDTA (1 ml/75 cm²; PAA Laboratories, L11-004) or accutase (1 ml/75 cm²; PAA, L11-007) until complete detachment was achieved (1-5 min). Cells were then suspended in complete DMEM or EBM and replated for use in assay.

2.10 Measurement of mRNA expression

2.10.1 Sample preparation

Murine aortae were lysed in TRIzol® reagent (Invitrogen 15596-026) by mechanical homogenisation (30 Hz, 2 min) with stainless steel beads (Qiagen 69989). Monolayers of bAEC, HUVEC and sEnd1 were washed with ice cold PBS and then lysed directly in TRIzol® reagent (1 ml/10 cm²). Total RNA was extracted using chloroform and a series of alcohol washes as per manufacturer's instructions, and purified by sodium acetate/ethanol precipitation. RNA was then reverse-transcribed to cDNA using Ready-to-Go You-Prime First-Strand Beads (GE Healthcare, 27-9264-01), as per manufacturer's instructions. Briefly, 2 µg RNA was brought to a volume of 30 µL in diethylpyrocarbonate (DEPC)-treated water, and heated at 65°C for 10 min. The RNA solution was then transferred to a tube of first-strand reaction mix beads and 1 µM Oligo(dT)20 primer (Life Technologies, AM5730G) was added. Samples were incubated at RT for 1 min. Tubes were then gently vortexed and incubated at 37°C for 1 h. DNA purity was assessed in a NanoDrop 1000 spectrophotometer (Thermo Scientific). Lysates of dorsal root ganglia (DRG) isolated from the same mice were prepared in a similar manner.

2.11 Primers and PCR conditions

TRPV1 cDNA was amplified using the following primer pairs:

1. **Murine TRPV1 (predicted product size: 246 bp)**
 - a. Forward: 5'-ctgatcgaggatgggaagaa-3'
 - b. Reverse: 5'-aatgagcatgttgagcagga-3'
2. **Bovine TRPV1 (predicted product size: 173 bp)**
 - a. Forward: 5'-gaatcacgggaatctcagga-3'
 - b. Reverse: 5'-gagcagtcctatgaggatgt-3'
3. **Human TRPV1 (predicted product size: 201 bp)**
 - a. Forward: 5'-caaccagctgggcatcgtgaagt-3'
 - b. Reverse: 5'-agcttcagcgtcgggtgcagttt-3'

TRPV4 cDNA was amplified using the following primer pairs:

1. **Murine TRPV4 (predicted product size: 172 bp)**
 - c. Forward: 5'-acaagaagcgctgactgat-3'
 - d. Reverse: 5'-tctctgaaggcgagttgat-3'
4. **Bovine TRPV4 (predicted product size: 245 bp)**
 - a. Forward: 5'-taccgaccacaatggacta-3'
 - b. Reverse: 5'-accaaggcaaagaccatgac-3'
5. **Human TRPV4 (predicted product size: 226 bp)**
 - a. Forward: 5'-aggtgtgcaatgaggaccagaccaa-3'
 - b. Reverse: 5'-atgagggcaatgagcatgttgagga-3'

Thermocycling was performed as described in Table 1. PCR products were visualised by 1% agarose gel electrophoresis (80 V, 50 min) and ethidium bromide staining under UV illumination.

2.12 Measurement of intracellular calcium

2.12.1 Microplate-based live imaging

2.12.1.1 Experimental set-up

Cells were plated in black 96-well plates (Corning, NY, USA) at an approximate density of 30,000 cells/well and allowed to adhere overnight. Cells were then loaded with 2.5 μ M Fura-2-acetoxymethyl ester (Fura-2/AM; Merck Chemicals, 344906) in the presence of 2 mM probenecid in Hank's balanced salt solution (HBSS, pH 7.4) containing Ca^{2+} , Mg^{2+} and 20 mM HEPES. After 1 h, the loading solution was replaced with HBSS, and fluorescence in each well was measured using a FlexStation® 3 Microplate Reader (Molecular Devices, Sunnydale, CA). Emission intensity ratios at 340 nm/380 nm excitation wavelengths (response ratio) were measured every 6 s for 180 s.

2.12.1.2 Cell treatments.

Prior to assay, cells were either left untreated, or were incubated with lipopolysaccharide (LPS; 100 ng/ml; 1–24h). Following 20 s measurement of baseline intracellular calcium levels, drugs were added by an internal automated pipettor system.

2.12.2 Single-cell live imaging

Cells were plated onto circular glass coverslips (13 mm diameter) and allowed to adhere overnight. Cells were incubated with 2.5 μ M Fura-2/AM and 2 mM probenecid in HBSS, in the dark at 37°C for 1 h. Coverslips were then mounted in a laminar flow perfusion chamber, and continuously perfused at a rate of 4 ml/min with either HBSS, HBSS + capsaicin, HBSS + GSK1016790A, or HBSS + positive control (either 1 μ M ionomycin or 100 μ M ATP). Baseline recordings were taken for 30 s, and drugs were perfused for 2 min and washed out for 2 min. The temperature of the perfusion buffer was maintained at 37°C by a regulated Peltier device (Marlow Industries, Dallas, TX) and monitored by a thermocouple. Images of a group of cells were captured every 2 s (excitation 340 nm and 380 nm; emission 520 nm) using a Zeiss Axiovert microscope, an ICCD video camera (Stanford Photonics, California, USA) and video microscopy acquisition software EasyRatioPro (Photon Technology International, New Jersey, USA).

2.12.3 Analysis

Analysis of emission intensity ratios at 340 nm/380 nm excitation (response ratio) in individual cells was performed with EasyRatioPro analysis software (Photon Technology International, USA). All cells non-responsive to positive control were excluded from analysis. Analysis of ratios in microplate wells was performed with Softmax Pro analysis software (Molecular

Devices, LLC, USA). Baseline fluorescence intensity was subtracted from readings post-drug administration in order to obtain relative response. A ratio change of 0.1 or greater was considered responsive (Alexander et al., 2013). All subsequent analysis was performed in Microsoft Excel and GraphPad Prism.

2.13 Assessment of cell viability by MTT assay

Cell viability was assessed by MTT assay, as described in greater detail in section 5.2.6. Cells were plated in 96-well plates and allowed to reach 80% confluency. 10 μ L MTT (in saline) was added to each well (final concentration: 5 mg/mL), and cells were incubated at 37°C for 4 h. The formazan product was solubilised in DMSO, and absorbance was read at 560 nm in a FlexStation® 3 Microplate Reader (Molecular Devices, Sunnydale, CA).

CHAPTER 3 | A NOVEL APPROACH TO MEASURING MURINE MESENTERIC BLOOD FLOW IN SITU

3.1 Introduction

While care of septic patients has traditionally centred on achieving target blood pressures and a normal macrohaemodynamic status, it is now becoming increasingly clear that monitoring systemic parameters does not reliably predict mortality: such assessments fail to reflect the status of the microcirculation, where impaired flow is associated with significant tissue damage and poor outcome (De Backer et al., 2010). Indeed, persistent disruption of microcirculatory perfusion following the restoration of macrohaemodynamic parameters has been shown to correlate strongly with organ failure and death (Sakr *et al.*, 2004).

This has been demonstrated in animal models of shock, as well as in human patients. Endotoxaemic sheep in which mean arterial pressure (MAP) was restored through noradrenaline infusion nevertheless exhibited mesenteric flow disruption and severe mitochondrial dysfunction (Andersson et al., 2012). Furthermore, in rats subjected to haemorrhagic shock and subsequent blood volume restoration, survival was found to be strongly related to the level of skeletal muscle perfusion, but not to macrohaemodynamic parameters (Zhao et al., 1985). Similarly, De Backer and colleagues have shown that sublingual circulation is compromised to a greater extent in non-survivors of septic shock than survivors (De Backer et al., 2010), and numerous studies have shown that elevating arterial pressure with vasopressors does not necessarily improve regional perfusion (Dubin et al., 2009, Jhanji et al., 2009, Gattinoni et al., 1995).

These observations have given rise to an emerging theory that systemic stabilisation in sepsis may actually be achieved at the expense of the microcirculation, where impaired flow leads to regional hypoxia and eventually multiple organ dysfunction syndrome, or MODS (Spronk et al., 2004). The exact mechanisms underlying microcirculatory shutdown in sepsis are not well understood; active shunting, microthrombus formation and endothelial dysfunction are all likely to contribute. What is clear, however, is that microcirculatory dysfunction is at the heart of sepsis-associated pathology (Spronk et al., 2004), and clinical practice has adapted in line with this growing realisation: assessment of microcirculatory perfusion (by measurement of proxies, such as blood lactate levels or gut tonometry) has become common clinical practice,

and recently direct measurement of sublingual blood flow by sidestream dark field imaging has emerged as a robust predictor of outcome in patients (Trzeciak et al., 2008, Spanos et al., 2010).

Despite this shift in clinical practice, however, pre-clinical models of sepsis rarely assess microcirculatory parameters directly, and where they have been evaluated, methods are often either exceedingly complex and invasive, and therefore limited to larger species, or subject to significant confounding factors, such as dissection-induced tissue damage. As such, it is clear that more relevant pre-clinical models are required, in order to increase understanding of the pathological microvascular changes that occur in septic shock. Owing to the ease of genetic modification in mice, it is particularly crucial to develop more clinically relevant models in this species, in order to gain specific mechanistic insight into the involvement of particular proteins in sepsis pathogenesis. Furthermore, a greater understanding of the relationship between haemodynamic variables (including blood pressure and cardiac output), microcirculatory perfusion and haematological indices or organ function and metabolic disturbances is crucial in this syndrome. Systematic multi-parameter modelling, with a focus on clinically relevant endpoints, would be immensely valuable for early proof-of-concept investigations, and could help to refine the development of novel treatment strategies.

3.1.1 Hypothesis

We hypothesise that microcirculatory flow is a more sensitive marker of sepsis progression than global haemodynamic parameters, and that the latter may be maintained at the expense of the former.

3.1.2 Aims

The aims of this study were as follows:

1. To establish a robust model of quantitative microcirculatory flow assessment in the mouse
2. To measure microcirculatory blood flow in healthy and septic mice, and to evaluate the physiological relationship between microvascular function, global haemodynamic variables and haematological markers
3. To determine the prognostic value of microcirculatory blood flow relative to global haemodynamic parameters, through reference to systemic markers of organ dysfunction
4. To determine the clinical relevance of using microvascular blood flow as a pre-clinical endpoint through use of standard clinical interventions
5. To elucidate the mechanisms of microcirculatory flow impairment in sepsis using vasodilatory and anti-coagulant agents

3.2 Methods

3.2.1 Experimental design

The mouse model is essential for delineating the involvement of specific proteins in sepsis pathogenesis. While it is clear that rodents do not necessarily recapitulate the genomic responses to sepsis encountered in humans (Seok et al., 2013), they nevertheless exhibit highly similar pathophysiological sequelae, most notably a profound inflammatory response with subsequent thermoregulatory and cardiovascular perturbations. As such, the mouse represents a useful tool for early proof-of-concept studies in a mammalian system that largely mirrors the sepsis-induced pathophysiological changes that occur in human patients.

The two most widely used pre-clinical models of sepsis and septic shock involve either administration of a bacterial endotoxin (typically lipopolysaccharide, or LPS) to stimulate systemic endotoxaemia, or cecal ligation and puncture (CLP) to induce polymicrobial peritonitis, as described previously (Rittirsch et al., 2009). Both are associated with comparative advantages and disadvantages. LPS is highly stable, ensuring reproducible administration and easy storage. Furthermore, the quantity of LPS infusion can be easily titrated to induce a highly reproducible inflammatory response. The immunological profile of the LPS model is dissimilar to the polymicrobial sepsis syndrome encountered clinically (Seok et al., 2013), however, perhaps as a result of the bypassing of opsonisation and bacterial processing steps; the model is also limited by its lack of infectious focus, and species differences in susceptibility to LPS (Schultz and van der Poll, 2002). CLP, conversely, represents a polymicrobial insult more similar to the abdominal peritonitis encountered in humans. It has a septic focus from which bacteria are disseminated, though exact and consistent titration of insult severity may be more difficult to achieve. Based on these advantages and disadvantages, as well as their wide use in sepsis research, both models were investigated for comparison in this study.

The murine mesenteric bed was chosen for assessment of microcirculatory function on the strength of several important factors, not least its accessibility and its significant contribution to peripheral vascular resistance. Low mesenteric flow is known to correlate strongly with multiple organ failure and mortality, both in animal models (Baykal et al., 2000) and in human patients (Takala, 1997); clinically, gut ischaemia is known as the ‘motor of multiple organ failure’ (Carrico et al., 1986), probably because impaired mesenteric blood flow is associated with intestinal hyperpermeability. This breakdown in barrier function facilitates the leakage of endotoxins and microorganisms into the lymphatic and cardiovascular circulation, which can

exacerbate the inflammatory response (Sautner et al., 1998). Furthermore, gastric perfusion is known to correlate well with sublingual perfusion (Marik, 2001) – a robust indicator of outcome in patients (De Backer et al., 2010). Consequently, we have developed a novel approach to quantifying mesenteric blood flow *in situ*, in both healthy and septic mice.

3.2.2 Animals

Male C57-BL/6 mice (10–14 weeks; 25–30 g) were bred in-house or purchased from Charles River (UK). Sepsis was induced by LPS or CLP, as described in section 2.3. Naïve mice were used as controls in the LPS model; sham-operated mice (in which the cecum was exteriorised but not ligated or punctured) were used as controls for CLP.

3.2.3 Laser speckle contrast imaging

3.2.3.1 Experimental set-up

Mice were anaesthetised under isoflurane (2% by air pump) and core temperature was recorded and controlled by a rectal probe coupled to a homeothermic heating mat. Mice were maintained at the temperature at which they initially presented. Hair was removed from the abdomen by electrical shaver, and a small midline incision was made. A portion of the small intestine was gently exteriorised onto a parafilm-coated heating mat, and was pinned out through the gut wall to expose the mesenteric vasculature, as shown in **Figure 10**. Care was taken not to stretch or puncture blood vessels, and if bleeding was seen to occur, mice were terminated immediately and excluded from analysis (approx. 5% of total). The exposed vascular bed was kept moist with saline (0.5 ml, aerosolised) pre-warmed to 37°C. An additional heating mat was placed over the anaesthetised animal.

Mesenteric blood flow was recorded using a moorFLPI full-field laser perfusion imaging system and review software (Moor Instruments, Devon, UK) either in naïve animals, or at 6 or 24 h after the induction of sepsis. The following acquisition modes and settings were used: high resolution capture (25 frames, 1 s/frame); exposure 20 ms; automatic gain; flux palette set at 0–5000; background threshold 60 flux units. The moorFLPI was positioned approximately 25 cm above the region of interest, and zoom and focus were adjusted appropriately to acquire high-resolution images of the region of interest. No further investigator intervention or microdissection was necessary. All assessment and analysis was performed in a blinded manner. Baseline recordings were taken over 5 min.

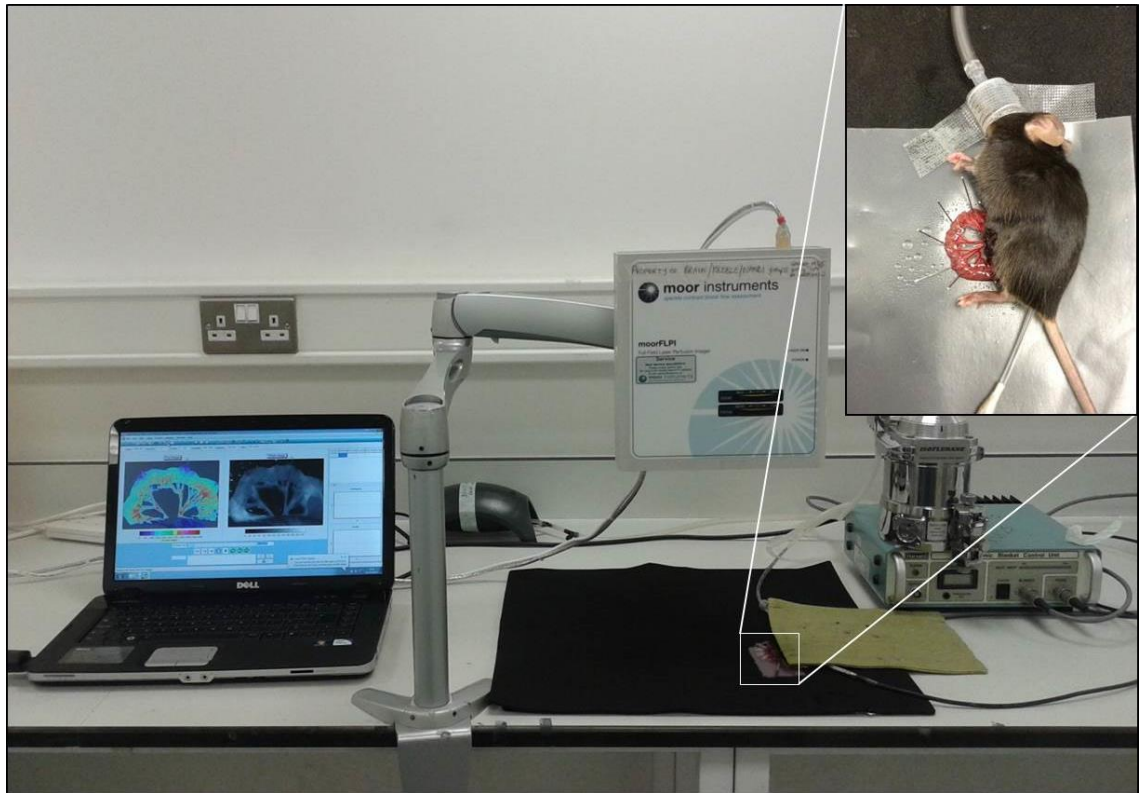


Figure 10. Experimental set-up involving exteriorisation of a section of the small intestine through a small abdominal incision, and careful pinning through the gut wall to expose the mesenteric vascular bed. Mice were placed on a parafilm-coated homeothermic blanket coupled to a rectal temperature probe. The exposed mesenteric bed was moistened with pre-warmed aerosolised saline solution, and an additional heating blanket was placed over the mouse prior to recording.

3.2.3.2 Theory of laser speckle contrast imaging

Laser speckle contrast imaging (LSCI) is based on the random speckle patterns produced when tissue is illuminated by laser light. In regions of high blood flow, the speckle pattern generated by moving red blood cells becomes blurred, reducing contrast in that region. Correspondingly, high contrast speckle patterns are associated with low flow (**Fig. 11**). Automated processing of the contrast images generates real-time, high-resolution, colour-coded flux images, as well as absolute flux values, which correlate with blood flow in each region of interest (Briers, 2001).

The excitation wavelength of incident light corresponds to the isobestic point of the absorption spectra of oxy- and deoxy-haemoglobin, such that emission is independent of the oxidative state of haemoglobin. This method confers several advantages over other methods of measuring microcirculatory perfusion:

1. Images are captured in real-time, allowing observation of very acute changes in blood flow. Traditional laser Doppler flowmetry techniques, by contrast, are associated with much slow processing speeds.
2. Images may be captured over a relatively large area, whereas laser Doppler and SDF probes are restricted to limited fields of view (often a single vessel), and are particularly sensitive to 'drift', which disrupts continuity of recording.
3. LSCI, using a full-field laser perfusion imager (moorFPLI), is not subject to user bias. The device is not hand-held, there is no contact between the probe and the vessel (eliminating the pressure artefact associated with SDF probes), and quantification is automated.
4. The technique does not require the fluorescent dyes necessary for intravital microscopy, nor does it require a high level of microdissection, eliminating numerous confounding factors.

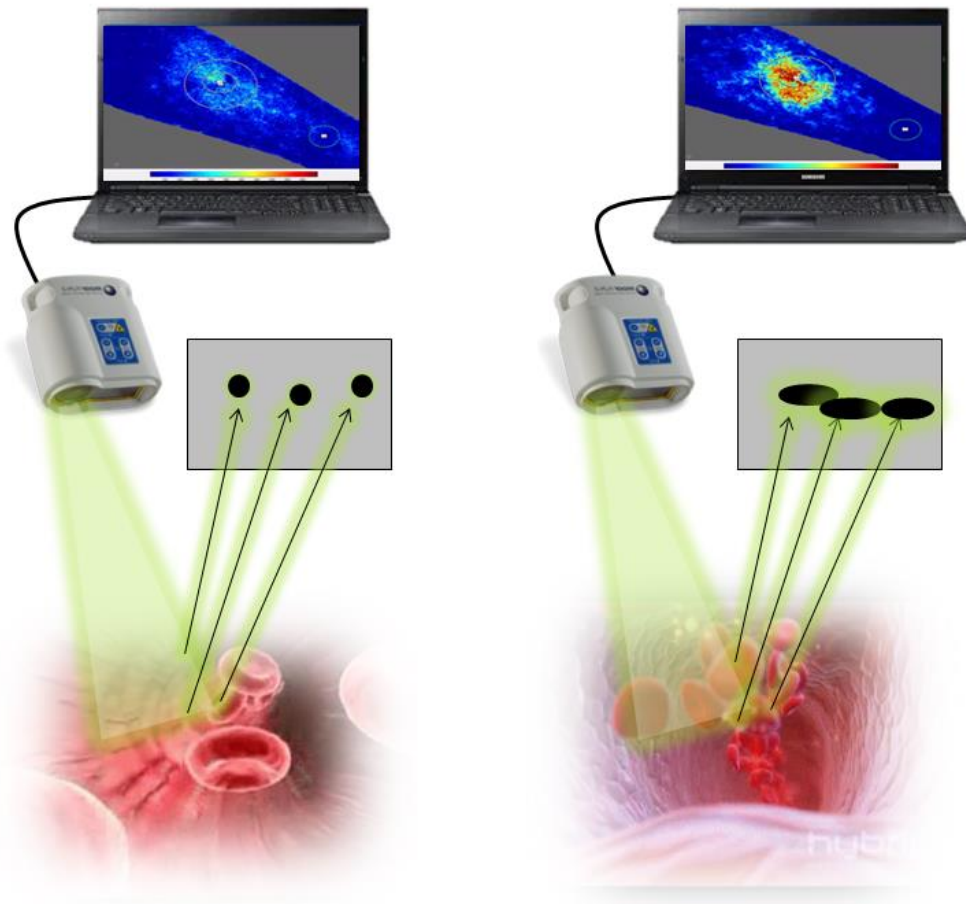


Figure 11. Laser speckle contrast imaging for quantification of blood flow. Left hand panel: illumination of slow-moving red blood cells generates a high-contrast speckle pattern, which is processed as a 'low blood flow' area (colder colours) on a colour-coded image. Right hand panel: illumination of fast-moving red blood cells generates a blurred speckle pattern, which is processed as 'high blood flow' (warmer colours). Quantification of absolute flux values is automated. Figure adapted from 'Medical Animation of Red Blood Cells', Tom Guthery; *Medical Animations*. Flix Productions (Guthery, 2007).

3.2.3.3 Blood flow analysis

3.2.3.3.1 Mesenteric blood flow

Flux over time was analysed offline by moorFLPI Review software (V3; Moor Instruments, Devon, UK) and vessels were designated as 1st, 2nd or 3rd order branches of the mesenteric vascular tree (**Fig. 12**). Regions of interest in which flux over time was measured were defined in each visible vessel, and a mean value was obtained for each level of mesenteric branching in each vascular bed. Results were analysed in moorFLPI Review software (V4.0), Microsoft Excel and GraphPad Prism. Results are expressed as mean area under the curve (AUC; flux units.time) \pm SEM.

3.2.3.3.2 Ear blood flow

A polygon tool was used to select the precise outline of both ears as regions of interest (**Fig. 13**) in moorFLPI Review software (V3). Mean flux across both ears was calculated for each mouse at each time point over a 3-min baseline recording using Microsoft Excel and GraphPad Prism. Results are expressed as mean area under the curve (AUC; flux units.time) \pm SEM.

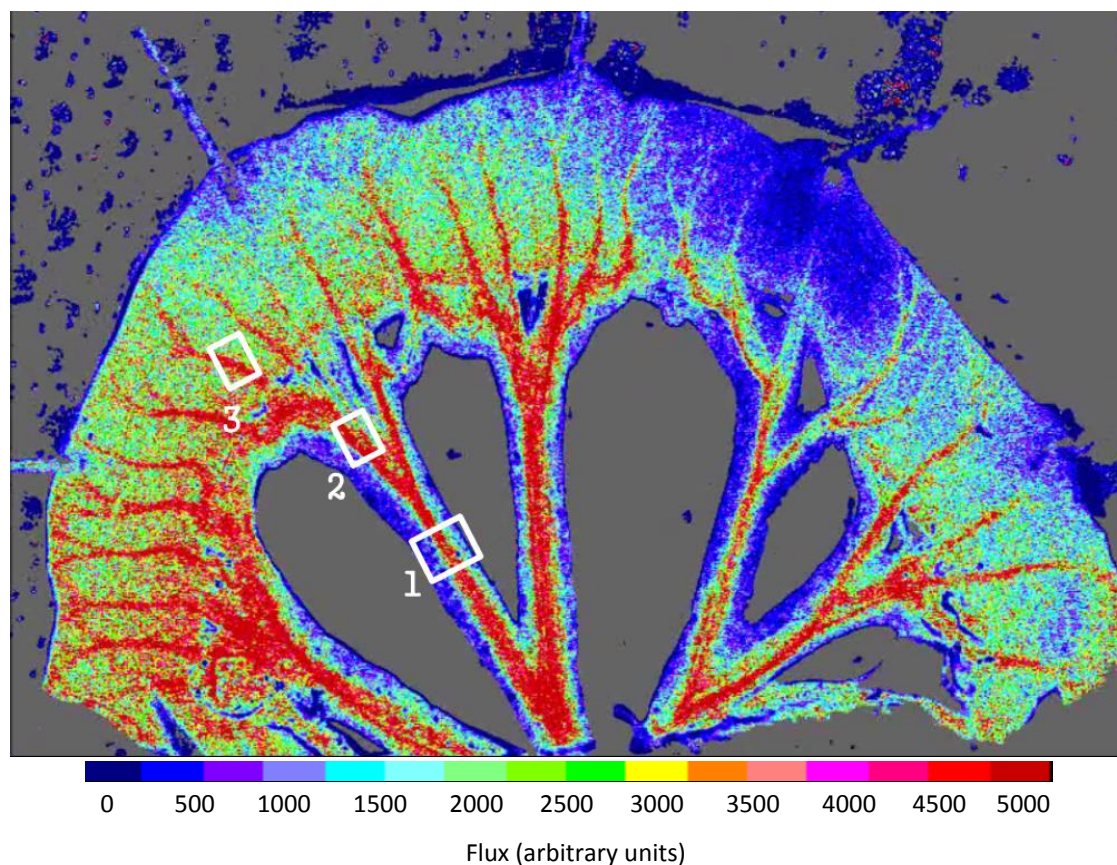


Figure 12. Regions of interest for quantification of mesenteric blood flow. Vessels are designated as 1st (1), 2nd (2), or 3rd (3) order branches of the mesenteric vascular tree. Regions of interest were placed as indicated by white boxes over each visible vessel, and a mean value of flux over time was calculated for each level of mesenteric branching.

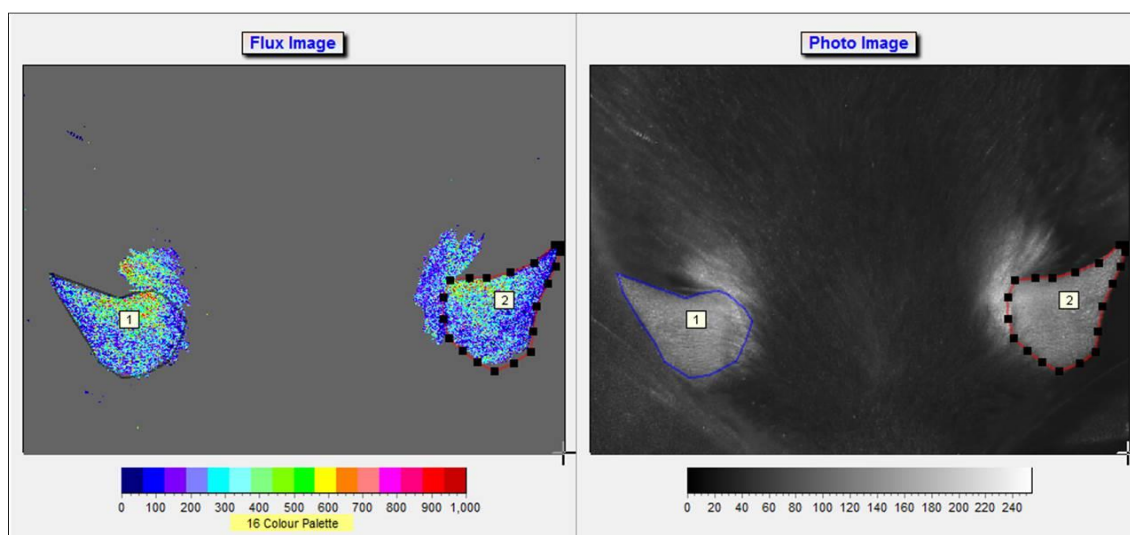


Figure 13. Regions of interest for assessment of subcutaneous ear blood flow. A polygon tool was used to draw the precise outline of each ear, in which flux over time was recorded. A mean flux values across both ears was calculated for each mouse.

3.2.4 Fluid resuscitation of septic animals

Aggressive fluid resuscitation is one of the only clinical interventions known to improve survival in septic patients (Rivers et al., 2001). In order to determine the clinical relevance of using mesenteric blood flow as an endpoint measure, a resuscitation protocol was employed in the more clinically relevant CLP model. Following CLP surgery, mice were randomised to receive either no intervention, or Hartmann's solution containing 5% dextrose (40 ml/kg, s.c.) at 0 h, 3 h, 6 h and 18 h post-sepsis (under brief isoflurane anaesthesia), based on a regimen known to attenuate mortality in septic mice (Zanotti-Cavazzoni et al., 2009). Control sham and non-resuscitated mice were briefly anaesthetised at identical time points, and a sham injection was administered. Mesenteric blood flow and blood biochemistry were assessed at 24 h post-CLP, as described in sections 3.2.1 and 3.2.6, respectively. Subcutaneous blood flow in the ear was also measured over a 3-min baseline with mice in the prone position, and settings identical to those described in section 3.2.1, with the exception of the flux palette, which was set at 0–1000.

3.2.5 Systemic administration of vasoactive agents

Further mechanistic studies were undertaken using the LPS model. In order to validate the use of the mesenteric blood flow model for assessment of acute vasoactive responses to systemically administered drugs, an intravenous catheter was introduced into the left jugular vein of LPS-treated mice under isoflurane anesthesia (2% by air pump), immediately prior to blood flow measurement at 24 h post-LPS. After a 5-min baseline recording, a bolus dose of saline (100 µl over 10 s) was administered intravenously, followed 5 min later by a bolus dose of noradrenaline bitartrate (30 µg/kg in saline; 100 µl over 10 s; Sigma-Aldrich). Responses to noradrenaline were recorded over a further 5 min.

3.2.6 Topical administration of vasoactive agents

The impact of a topical vasodilator was also assessed in LPS-treated mice, in order to determine reversibility of mesenteric flow perturbations. After 5 min baseline, recording was paused and sodium nitroprusside (SNP; 10 µM in saline, pre-warmed to 37°C) was administered to the mesentery as an aerosolised spray (2 pump compressions, approx. 200 µl, from a distance of 10 cm). Recording was immediately resumed for a further 5 min.

An aerosolised delivery method was chosen to prevent interference with laser reflection by large droplets of liquid, and to ensure even coverage of the exposed vascular bed.

3.2.7 Systemic anticoagulant administration

Given that microcirculatory flow impairment is likely to reflect a combination of disseminated intravascular coagulation and arterio-venous shunting, we aimed to elucidate the relative contributions of both factors by pre-treating septic mice with systemic anticoagulant therapy. Heparin (100 U/kg in 100 µl saline, i.p.; Sigma) was administered 5 min prior to the induction of sepsis by LPS or CLP, as described in section 2.3.

3.2.8 Termination and analysis of blood biochemistry

Following mesenteric blood flow recording, a venous blood sample was drawn from the inferior vena cava, and animals were terminated by cervical dislocation. Blood biochemistry was assessed immediately from 100 µl venous blood using a hand-held iSTAT® point-of-care analyser (Abbott Laboratories, IL, USA), with CG8+ cartridges (Abbott Laboratories). In a small sub-set of experiments, an arterial blood sample was drawn from the left ventricle of the heart, in addition to a venous sample, for comparison of arterial and venous blood composition. For fluid resuscitation experiments additional CG4+ cartridges (Abbott Laboratories) were used for quantification of blood lactate levels.

3.2.9 Acute thrombosis modelling

In order to assess other potential applications of the mesenteric blood flow model, changes in blood flow were measured in response to an acute thrombotic stimulus. Mice were set-up as described in section 3.2.2.1. After a 3-min baseline, recording was paused and a 1-mm² square of filter paper soaked either in FeCl₃ (20% in saline) or saline was applied to a chosen vessel for 10 s. The filter paper was then removed, and recording was resumed for a further 30 min. This model is thought to induce a 'mixed thrombus', composed of fibrin, activated platelets and erythrocytes (Lockyer and Kambayashi, 1999). On a microscopic level, FeCl₃-induced thrombosis is associated with separation of endothelial cell junctions and endothelial denudation, which in turn leads to platelet adherence and activation, thrombin generation and activation of the coagulation cascade (Kurz et al., 1990).

3.2.10 Multi-parameter monitoring in murine sepsis models

Mice were implanted with telemetry probes, and cardiac function was assessed via echocardiography by Dr Anna Starr, as described in sections 2.4 and 2.5. A multi-parameter monitoring system encompassing haemodynamic, microcirculatory and haematological assessment (shown schematically in **Figure 14**) was developed to allow comprehensive cardiovascular assessment in a single mouse, facilitating paired comparison.

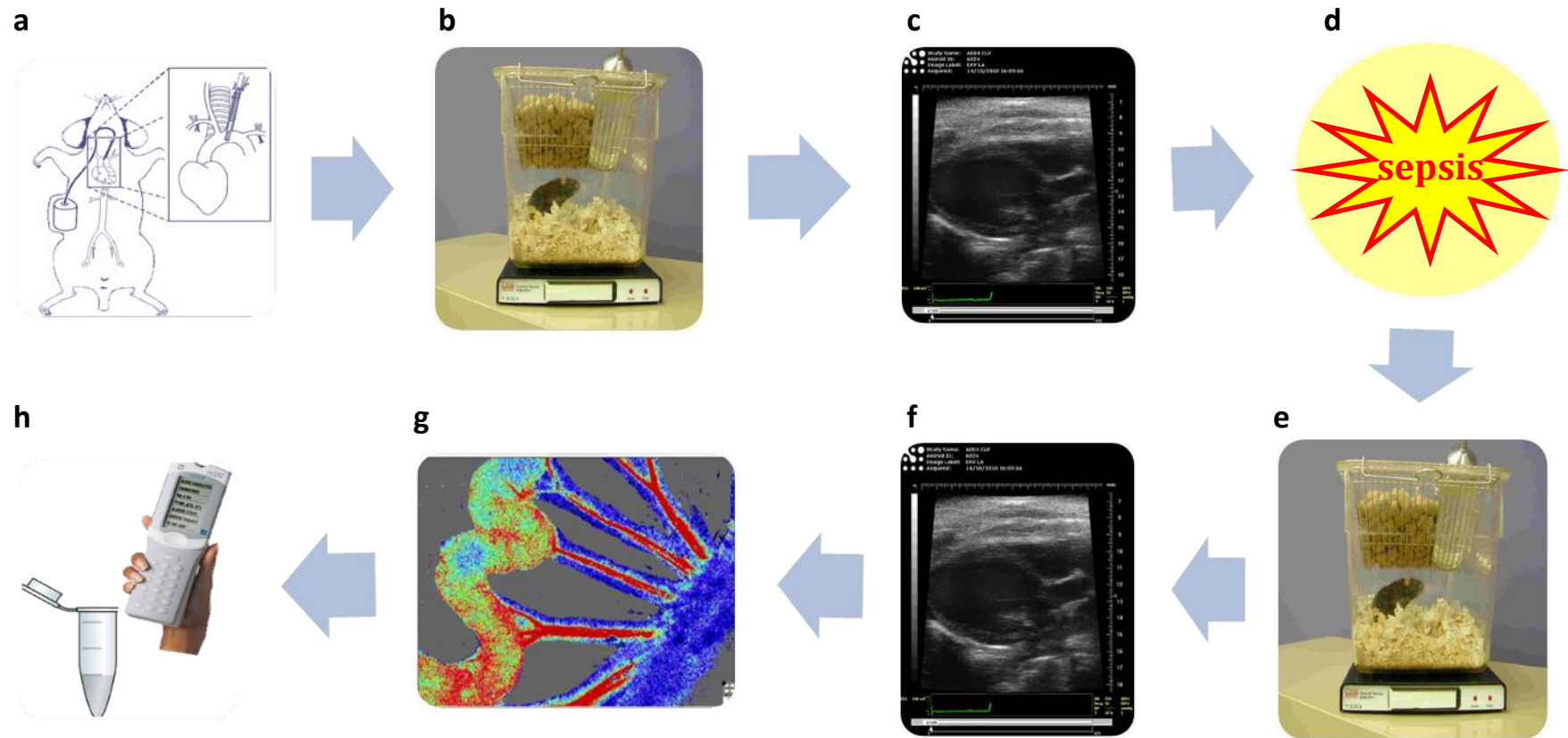


Figure 14. Schematic representation of multi-parameter monitoring of sepsis progression in a single mouse. Following implantation of a telemetry catheter (a), baseline haemodynamics are recorded remotely in unrestrained ambulatory mice (b). Basal cardiac function is measured under isoflurane anaesthesia by echocardiography (c). Sepsis is then induced either by LPS or CLP (d), and haemodynamics (e) and cardiac function (f) are re-recorded in the same mice under pathological conditions. Mice are then terminally anaesthetised at a time point of interest, and mesenteric blood flow is measured by laser speckle contrast imaging (g). Intravenous or topical drug administration may be used to determine vasoactivity. Mice are then terminated by venupuncture, and blood samples obtained from the inferior vena cava are analysed by iSTAT point-of-care analyser (h). Further biochemical and histological investigation may then be carried out.

3.3 Results

3.3.1 Mice anaesthetised for mesenteric blood flow assessment are homeothermically and haemodynamically stable.

Core temperature (**Fig. 15**) and mesenteric blood flow (**Fig. 16**) were found to remain stable over the recording period (and for up to 60 min) in control and septic mice, validating the use of this model for investigating acute responses to vasoactive stimuli, independent of fluctuations in temperature or haemodynamic status. LPS-treated mice exhibited a hypothermic phenotype at 6 h that was further exaggerated by 24 h. CLP-treated mice became gradually, but less severely, hypothermic over 24 h. These observations are consistent with previous reports (Clark et al., 2007, Coldewey et al., 2013, Saito et al., 2003).

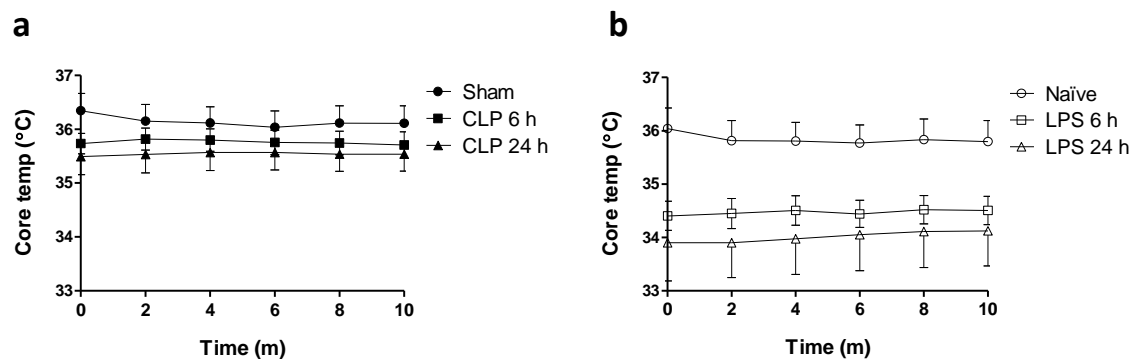


Figure 15. Core temperature, monitored by rectal probe, remained stable throughout the recording period. CLP-treated mice (**a**) were slightly more hypothermic than sham-operated mice, though hypothermia was more marked in LPS-treated mice (**b**), where core temperature dropped in a time-dependent manner following the induction of sepsis. Data are presented as mean \pm SEM, $n = 10$ –21.

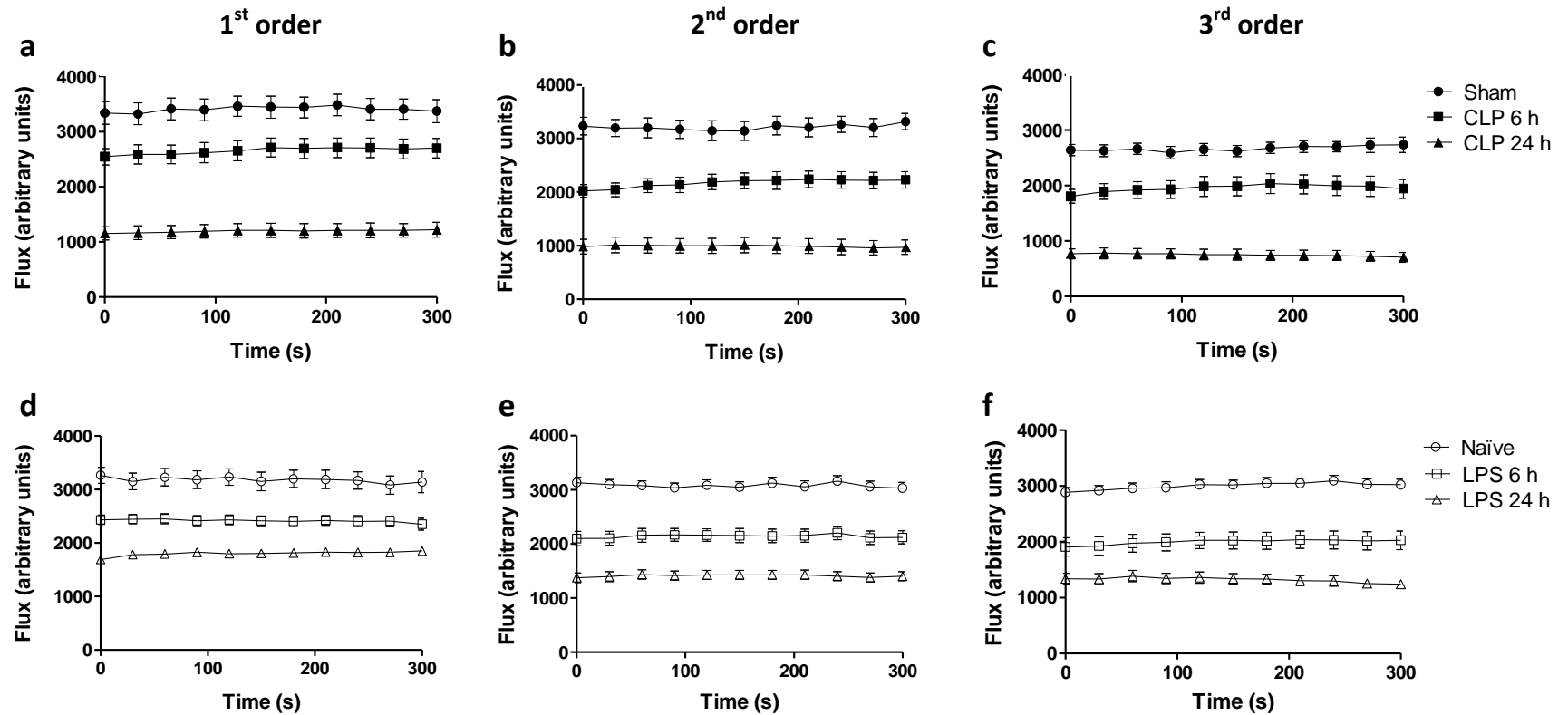


Figure 16. Absolute flux values recorded over 5-min baseline in healthy and septic mice. Top panel: flux values in sham- or CLP-operated mice (6 h or 24 h post-surgery) in (a) 1st, (b) 2nd, and (c) 3rd order mesenteric vessels. Bottom panel: flux values in naïve or LPS-treated mice (6 h or 24 h post-injection) in (d) 1st, (e) 2nd, and (f) 3rd order mesenteric vessels. Absolute flux values were found to be stable over recording period in both models of sepsis and in all vessel types. Data are presented as mean \pm SEM, n = 10–21.

3.3.2 Mesenteric blood flow declines in a time-dependent manner in both models of sepsis

Blood flow was significantly reduced in a time-dependent manner in both models of sepsis (**Fig. 17**). In the CLP model, blood flow was reduced by a 20–30% range at 6 h, and by a 65–70% range at 24 h post-sepsis; in LPS-treated mice, it was reduced by 25–35% at 6 h, and by 40–55% at 24 h.

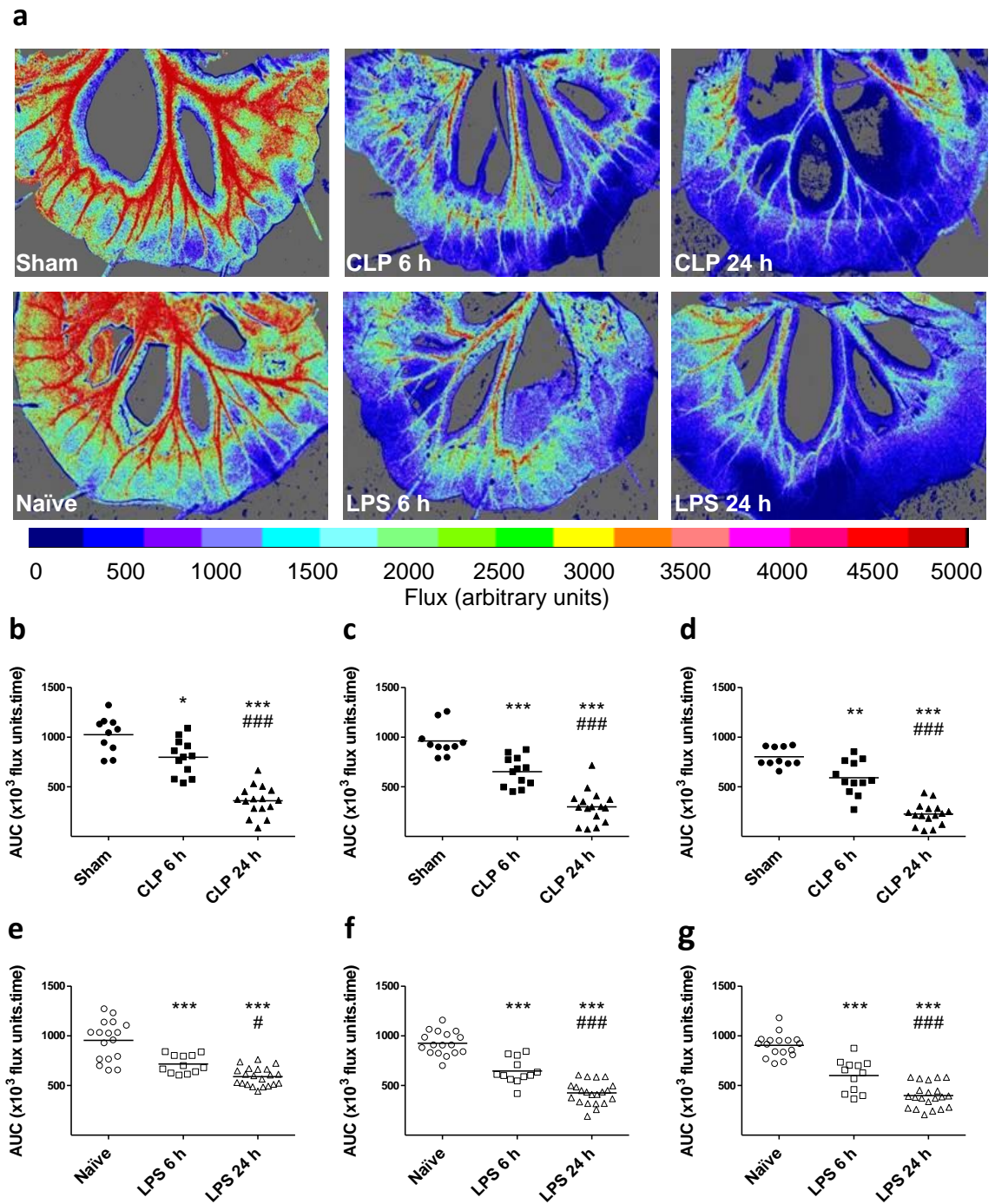


Figure 17. Mesenteric blood flow in healthy and septic mice. (a) Representative flux images taken during baseline recording. Cold colours indicate low flow, warmer colours higher flow. (b–d) Mesenteric blood flow in CLP model expressed as total area under the curve (AUC; $\times 10^3$ flux units.time) over 5 min baseline recording in 1st, 2nd and 3rd order vessels, respectively. (e–g) Mesenteric blood flow in LPS model expressed as total area under the curve (AUC; $\times 10^3$ flux units.time) over 5-min baseline recording in 1st, 2nd and 3rd order vessels, respectively. AUC for each animal is represented as an individual symbol, with horizontal line denoting group mean. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ relative to naïve/sham; # $p < 0.05$, ### $p < 0.001$ relative to 6 h time point, 1-way ANOVA + Bonferroni post-hoc test, $n = 10$ –21.

3.3.2.1 Comparison of mesenteric perfusion with indices of global haemodynamics

Mesenteric blood flow data was analysed in parallel with haemodynamic data. As shown in **Fig. 18**, mean arterial pressure (MAP), heart rate (HR) and locomotor activity, recorded by radiotelemetry, as described in section **2.4**, followed normal diurnal variation prior to the induction to sepsis (**Fig. 18; left hand panel**). Following induction of sepsis, LPS-treated mice became steadily more hypotensive, while mice subjected to CLP showed a more gradual decrease in MAP that was less marked than in the LPS model (**Fig. 18; right hand panel**). This likely reflects the accelerated nature of the LPS model, which bypasses bacterial processing and opsonisation steps, and is consistent with previous haemodynamic assessments in murine endotoxaemia (Rees et al., 1998). In both models, however, MAP was found to stabilise after 9 h, with equivalent values at 6 h and 24 h post-sepsis (CLP mean \pm SEM: 91.8 ± 2.6 mmHg vs 86.4 ± 5.3 mmHg at 6 h and 24 h, respectively, $n = 11$, ns; LPS mean \pm SEM: 76.4 ± 8.3 mmHg vs 77.0 ± 6.5 mmHg at 6 h and 24 h, respectively, $n = 11$, ns). Cardiac output, measured by echocardiography, was similarly decreased at 6 h post-sepsis in both models, but did not decline significantly thereafter (CLP mean \pm SEM: 19.7 ± 1.5 ml/min vs 18.3 ± 1.6 ml/min at 6 h and 24 h, respectively, $n = 8$, ns; LPS mean \pm SEM: 22.0 ± 1.9 ml/min vs 22.0 ± 1.1 ml/min at 6 h and 24 h, respectively, $n = 8$, ns). Conversely, mesenteric blood flow was found to decline continuously in both models of sepsis, and was significantly diminished at 24 h post-sepsis compared with the 6 h time point (**Fig. 17b–g**).

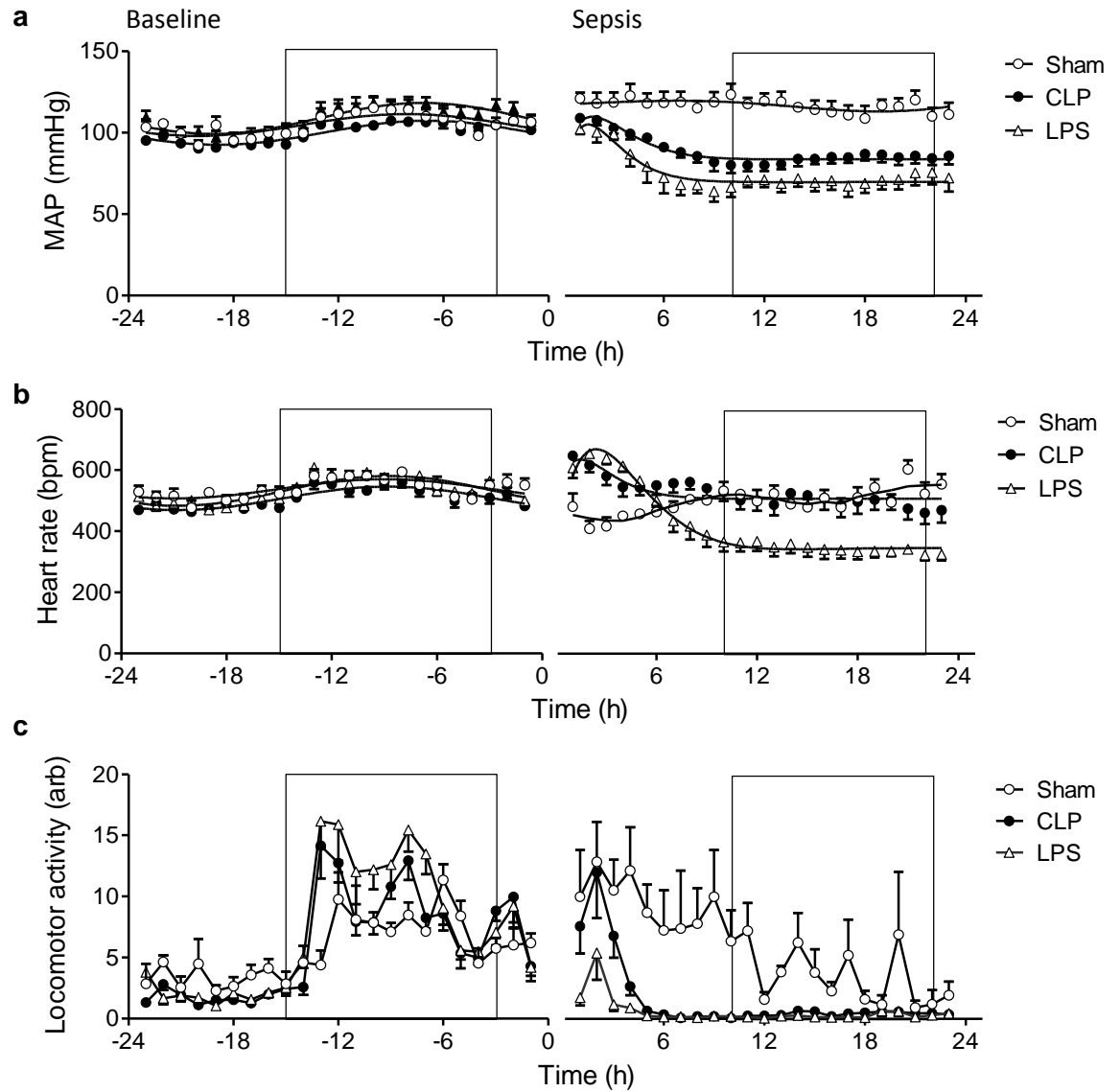


Figure 18. Assessment of systemic haemodynamics and locomotor activity in the same mice under naïve and septic conditions. (a) Mean arterial pressure, **(b)** heart rate and **(c)** locomotor activity, before and after induction of sepsis (at time 0: LPS, 12.5mg/kg or cecal ligation and puncture, CLP) or sham procedures. Boxed regions denote periods of darkness. Data were acquired by radiotelemetry in conscious, ambulatory male C57BL/6 mice by Dr Anna Starr, and are presented as mean \pm SEM, n = 6–11.

3.3.3 Markers of organ dysfunction and metabolic acidosis increase in line with reduced mesenteric perfusion

Consistent with impaired microcirculatory perfusion, blood urea was found to be markedly elevated at 24 h post-LPS and -CLP, compared with the 6-h time point (**Table 6**). A waste product of protein and amino acid metabolism, urea production can be variable and is largely dependent on protein intake. Since food intake was largely abolished following the induction of sepsis (as described more extensively in **Chapter 6**), this is unlikely to account for the changes in urea observed here. Reabsorption of urea from the proximal and distal nephron is known to be substantial in conditions of reduced renal perfusion (Rosner and Bolton, 2006), which likely explains the elevated blood levels measured here.

Blood pH was also significantly reduced in a time-dependent manner in both models of sepsis (**Table 6**). Both partial pressure of CO₂ (PCO₂) and bicarbonate levels were decreased by 6 h post-LPS and -CLP, indicative of metabolic acidosis. By 24 h, however, PCO₂ in CLP-treated mice became elevated (albeit non-significantly), consistent with respiratory acidosis. Venous oxygen saturation (SO₂) did not change significantly over the course of sepsis in either model, however. Mice also became severely hypoglycemic over the 24-h time period, indicative of metabolic disturbance similar to that observed in humans subjected to experimental endotoxemia (van der Crabben et al., 2009), and haematocrit and haemoglobin levels were also reduced following the induction of sepsis. These data and all other blood biochemistry are summarised in **Table 6**.

Consistent with metabolic acidosis the arterio-venous PCO₂ gradient had increased at 24 h post-CLP relative to sham-operated animals (mean Δ PCO₂ \pm SEM: 14.23 \pm 4.13 mmHg vs 1.90 \pm 1.30 mmHg, respectively, n = 4).

	Sham	6 h CLP	24 h CLP	Naïve	6 h LPS	24 h LPS
Urea (mmol/L)	4.79 ± 0.29	6.08 ± 0.67	17.03 ± 4.26*	4.57 ± 0.37	8.10 ± 1.45	36.47 ± 2.89***,###
pH	7.292 ± 0.01	7.225 ± 0.02	7.149 ± 0.03**	7.286 ± 0.03	7.245 ± 0.02	7.125 ± 0.03**
Base excess (mmol/L)	-11.87 ± 0.80	-17.00 ± 0.97	-15.21 ± 1.19	-11.0 ± 0.78	-12.60 ± 1.72	-17.19 ± 1.22***
HCO₃⁻ (mmol/L)	15.06 ± 0.68	10.67 ± 0.90	13.59 ± 0.84	16.89 ± 0.58	14.62 ± 1.80	12.53 ± 0.98
PCO₂ (mmHg)	30.91 ± 1.32	25.80 ± 2.12*	40.96 ± 4.73	39.94 ± 2.60	33.24 ± 3.93**	41.49 ± 3.72
TCO₂ (mmol/L)	15.93 ± 0.75	11.67 ± 1.02	15.50 ± 0.68	18.44 ± 0.93	15.80 ± 1.93	13.71 ± 1.06**
SO₂ (%)	84.43 ± 2.80	83.67 ± 3.84	84.11 ± 3.36	84.00 ± 3.67	88.00 ± 5.29	78.50 ± 3.43
Glucose (mmol/L)	13.24 ± 0.87	4.17 ± 0.62***	2.68 ± 0.40***	9.45 ± 1.65	2.27 ± 0.66***	2.20 ± 0.34***
Na⁺ (mmol/L)	147.3 ± 0.83	150.3 ± 0.88	151.9 ± 0.01**	152.6 ± 1.15	155.7 ± 0.88	152.8 ± 0.89
K⁺ (mmol/L)	4.122 ± 0.21	3.067 ± 0.15	4.167 ± 0.40	3.425 ± 0.43	3.233 ± 0.43	3.608 ± 0.36
Cl⁻ (mmol/L)	121.7 ± 1.07	127.3 ± 0.88	126.3 ± 1.46	109.0 ± 2.08	130.0 ± 1.53	124.7 ± 3.68
Anion gap (mmol/L)	15.67 ± 0.47	16.67 ± 0.67	16.78 ± 1.88	16.67 ± 0.69	17.00 ± 00	24.00 ± 3.08**
Haemoglobin (g/dl)	11.71 ± 0.51	10.47 ± 0.99	9.84 ± 0.60	12.39 ± 0.75	13.25 ± 1.92	10.33 ± 0.61
Haematocrit (%PCV)	33.11 ± 1.12	25.33 ± 2.96	29.67 ± 0.88	35.50 ± 2.18	27.00 ± 2.52	28.78 ± 1.85

Table 6. Blood biochemistry in healthy and septic mice. Blood gas and biochemistry were measured from venous blood samples by iSTAT point-of-care analyser in naïve and sham-operated animals, and at 6 h and 24 h post-LPS and -CLP. Data are presented as mean ± SEM. *p<0.05, **p<0.01 and ***p<0.001 relative to sham/naïve; ###p<0.001 relative to 6-h time point, 1-way ANOVA and Bonferroni post-hoc test, n = 3–16.

Abbreviations: PCO₂, partial pressure of CO₂; TCO₂, total carbon dioxide; SO₂, venous oxygen saturation; PCV, packed cell volume.

3.3.4 Assessment of mesenteric perfusion can be conducted in concert with haemodynamic analysis

The presence of a telemetry probe in the flank of a mouse had no bearing on the absolute flux values recorded. This allows measurement of both mesenteric blood flow and haemodynamic performance in the same mouse, facilitating direct comparison between the two parameters. The non-parallel relationship between microcirculatory perfusion and global haemodynamics is demonstrated clearly in **Figure 19**, which suggests that normalization of arterial pressure may be achieved at the expense of the microcirculation.

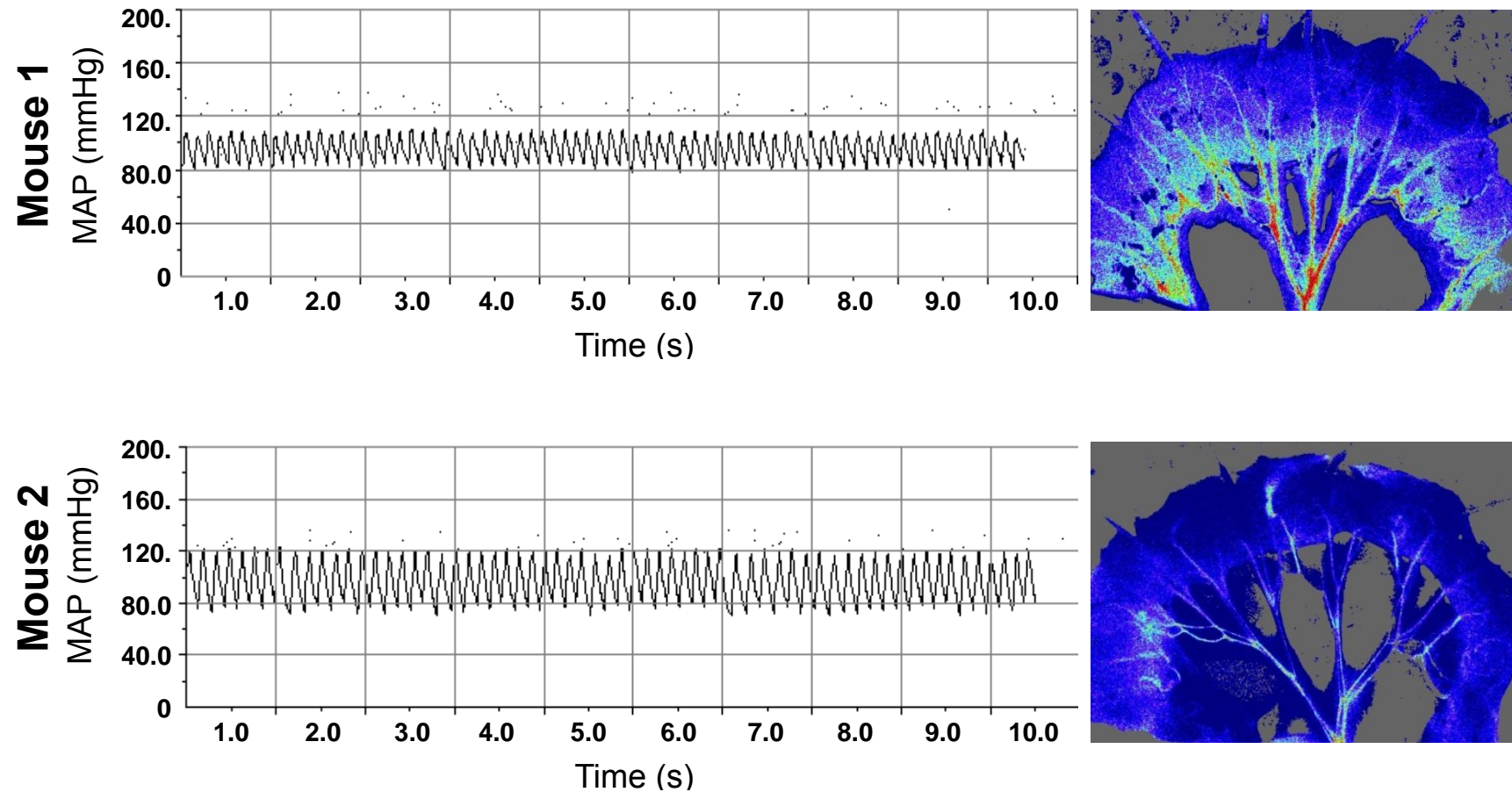


Figure 19. Representative blood pressure traces and corresponding mesenteric blood flow images from two LPS-treated mice. Blood pressure traces were collected immediately prior to blood flow recording at 24 h post-LPS. Whereas Mouse 1 exhibited compromised mean arterial pressure (MAP) and pulse pressure, mesenteric perfusion was less severely disrupted relative to Mouse 2, which exhibited a profound decrease in mesenteric perfusion, despite normal pressure. This demonstrates that MAP alone is insufficiently predictive of syndrome progression.

3.3.5 Fluid resuscitation attenuates CLP-induced microvascular disturbance

Given the continued deterioration of mesenteric blood flow over the 24-h period, compared with the apparent preservation of macrocirculatory haemodynamics (**Fig. 18**) and global oxygen saturation (**Table 7**), we aimed to ascertain whether sepsis-induced impairment of microcirculatory blood flow was sensitive to fluid resuscitation – a mainstay clinical intervention known to improve microcirculatory perfusion, organ function and survival in patients (Rivers et al., 2001) and septic mice (Hollenberg et al., 2001).

This intervention modestly, but significantly, improved perfusion in 1st order mesenteric vessels (**Fig. 20a**), and a similar pattern of improvement was observed in 2nd and 3rd order vessels [mean area under the curve (AUC; $\times 10^3$ flux units.time): 220.9 ± 52.3 vs 406.7 ± 78.0 in 2nd order vessels, ns; 140.8 ± 33.5 vs 342.9 ± 44.1 in 3rd order vessels, $p < 0.05$]. Volume-resuscitated mice also exhibited modest improvements in hypothermia (**Fig. 20d**), arbitrary severity score (based on posture, mobility, aversion to touch and piloerection, assigned in a blinded fashion; **Fig. 20c**), metabolic acidosis, and urea and lactate production (**Table 7**). Interestingly, measurement of perfusion by the same method in a different microvascular bed – the ear – revealed a significant decrease in blood flow in CLP-treated mice that was also reversed by fluid resuscitation (**Fig. 20b&e**). However, sepsis-associated hypernatraemia, evident in CLP-treated mice, was found to be exaggerated by fluid resuscitation (probably due to the crystalline nature of the fluid). The small decrease in haematocrit observed in unresuscitated CLP-treated mice was exaggerated in resuscitated mice, likely as a consequence of fluid loading and intravascular volume expansion.

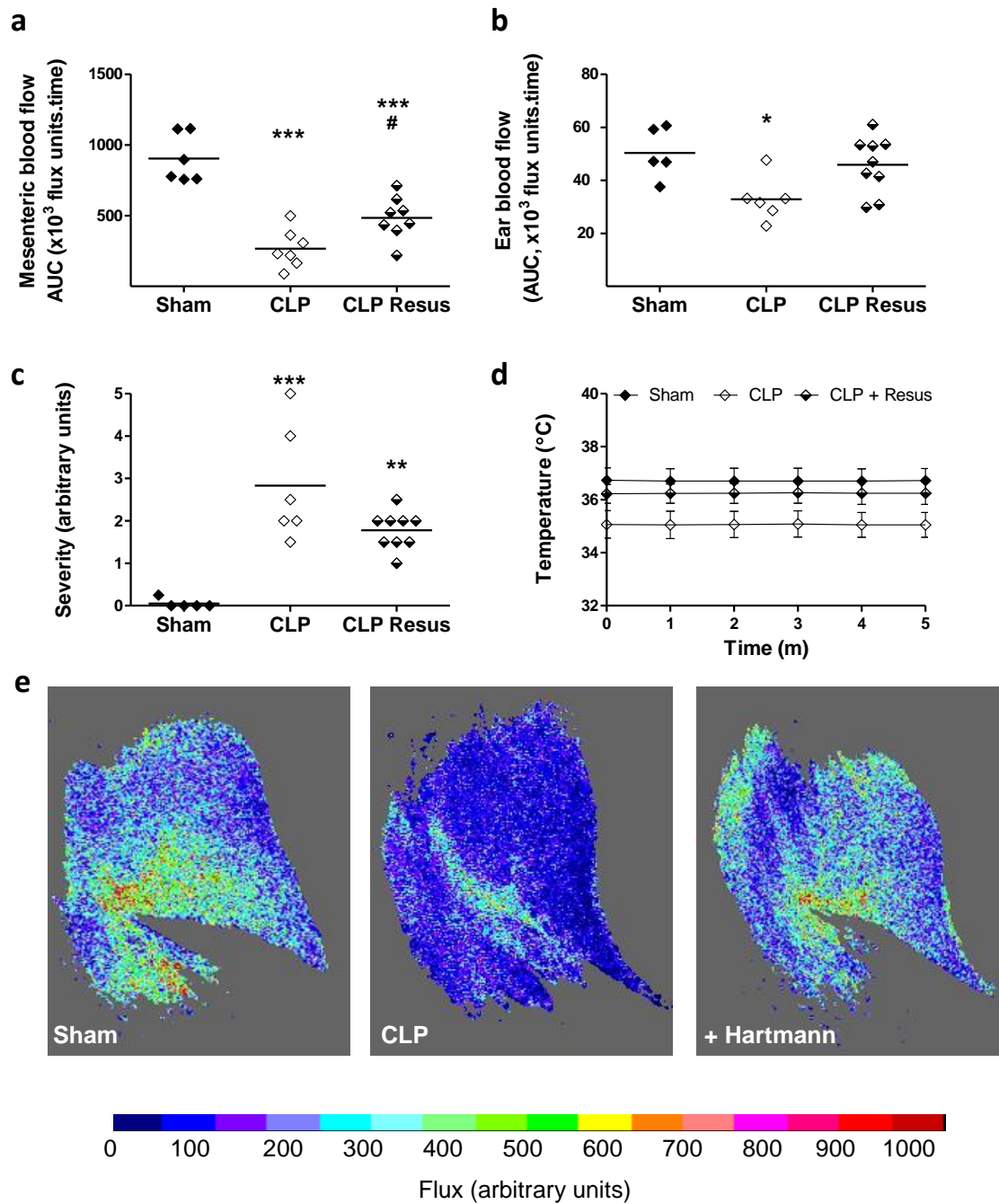


Figure 20. Impact of Hartmann's fluid resuscitation on sepsis parameters. Hartmann's resuscitation (40 mg/kg, s.c.; Resus) was administered immediately after CLP and at 3 h, 6 h and 18 h thereafter. Control sham- and CLP-treated animals were given a bolus injection immediately post-surgery but received sham injections at later corresponding time points. **(a)** Blood flow in 1st order mesenteric vessels expressed as area under the curve (AUC) over 5-min baseline recording. **(b)** Subcutaneous ear blood flow expressed as AUC over 3-min baseline recording. Data for individual animals are represented as individual symbols ($n = 5-9$), with group mean denoted by horizontal line. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, relative to sham, # $p < 0.05$, relative to CLP, 1-way ANOVA + Bonferroni post-hoc test. **(c)** Severity score (scale 0–5, arbitrary units) assigned in a blinded fashion based on voluntary mobility, gait, aversion to touch, facial expression and piloerection. **(d)** Core temperature measured by rectal probe during blood flow recording period. Data are presented as mean \pm SEM ($n = 5-9$). **(e)** Representative flux images of subcutaneous ear blood flow. Cold colors represent areas of low blood flow; warmer colors, areas of high blood flow.

	Sham	CLP	CLP Resus
Urea (mmol/L)	4.40 ± 0.33	15.82 ± 6.42	6.55 ± 0.99
pH	7.309 ± 0.02	7.126 ± 0.05**	7.220 ± 0.02
Base excess (mmol/L)	-10.80 ± 0.97	-15.17 ± 1.58	-13.25 ± 1.10
HCO₃⁻ (mmol/L)	15.52 ± 0.79	13.97 ± 1.04	14.36 ± 1.10
PCO₂ (mmHg)	30.94 ± 1.85	43.42 ± 5.99	35.56 ± 3.37
TCO₂ (mmol/L)	16.4 ± 0.81	15.17 ± 1.17	15.63 ± 1.24
SO₂ (%)	88.00 ± 2.61	88.20 ± 2.18	89.50 ± 1.23
Glucose (mmol/L)	13.50 ± 0.95	3.07 ± 0.57***	3.40 ± 0.35***
Na⁺ (mmol/L)	147.0 ± 0.89	151.3 ± 1.05*	154.8 ± 0.86***.#
K⁺ (mmol/L)	4.50 ± 0.14	4.68 ± 0.47	3.44 ± 0.12*.#
Cl⁻ (mmol/L)	120.0 ± 1.03	124.5 ± 1.77	128.1 ± 1.19**
Anion gap (mmol/L)	15.20 ± 0.73	17.67 ± 2.84	15.88 ± 0.88
Haemoglobin (g/dl)	11.70 ± 0.56	9.82 ± 1.34	9.49 ± 0.56
Haematocrit (%PCV)	34.14 ± 1.16	31.27 ± 1.48	27.88 ± 1.65*
Lactate (mmol/L)	3.92 ± 0.25	4.94 ± 0.51	3.61 ± 0.28 [#]

Table 7. Impact of fluid resuscitation on blood biochemistry in CLP model of sepsis. Blood gas and biochemistry measured from venous blood samples by iSTAT point-of-care analyser in sham-operated animals, and at 24 h post-CLP either with (Resus) or without repeated fluid resuscitation with Hartmann's solution. Data are presented as mean ± SEM. **p<0.01 and ***p<0.001 relative to sham; [#]p<0.05 relative to CLP, 1-way ANOVA and Bonferroni post-hoc test, n = 5–9. Abbreviations: PCO₂, partial pressure of CO₂; TCO₂, total carbon dioxide; SO₂, venous oxygen saturation; PCV, packed cell volume.

3.3.6 Intravenous administration of noradrenaline does not increase mesenteric perfusion in endotoxaemic mice

Intravenous bolus administration of noradrenaline, known to raise mean arterial pressure in endotoxaemic mice (Rees et al., 1998) and humans (Jhanji et al., 2009), did not increase mesenteric perfusion in either naïve or LPS-treated mice. On the contrary, it caused a marked decrease in blood flow (**Fig. 21**) that was comparatively sustained in LPS-treated mice. These data support the notion that improvement in global haemodynamic parameters does not necessarily result in improved regional perfusion, and that the pharmacological activity of vasopressors such as noradrenaline (α_1 adrenoceptor-mediated smooth muscle constriction) may override the physiological effects of increasing systemic arterial pressure.

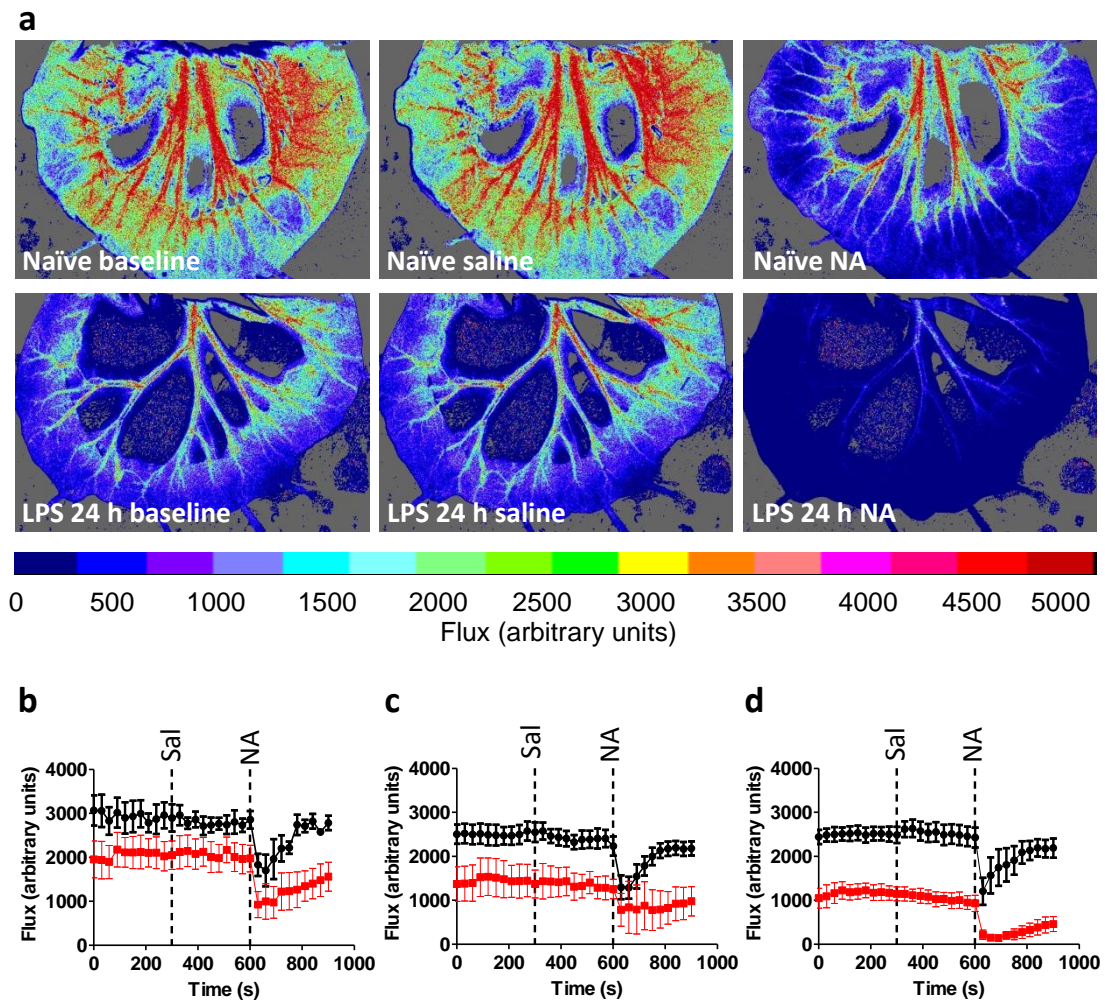


Figure 21. Effect of systemic administration of noradrenaline on blood flow in naïve and septic mice. (a) Representative flux images at baseline and following intravenous administration of saline (100 µl) and noradrenaline (NA; 30 µg/kg in 100 µl saline). Cold colours indicate low flow; warmer colours, high flow. Blood flow over time in (b) 1st order, (c) 2nd order, and (d) 3rd order branches of the mesenteric vascular tree. Following a 5-min baseline recording, saline was administered systemically, via an intravenous catheter in the left jugular vein, followed by a bolus injection of NA 5 min later. Black circles represent naïve mice; red squares, septic mice (12.5 mg/kg LPS, i.v., 24 h). Data are presented as mean ± SEM, n = 5.

3.3.7 Topical administration of a vasodilatory agent reverses the decrease in mesenteric blood flow induced by LPS

In order to test our hypothesis that the mesenteric bed is actively shut down in order to preserve global circulation, we applied sodium nitroprusside (SNP), a nitric oxide donor, directly to the exposed mesenteric vasculature by aerosolised spray. 10 μ M SNP was found to reverse LPS-induced microvascular impairment in all order branches of the mesenteric vascular tree (**Fig. 22**). These data suggest that active vasoconstriction accounts, at least in part, for the reduced mesenteric perfusion observed in sepsis, and are consistent with the effects of topical vasodilators applied to the sublingual microvasculature in septic patients (De Backer et al., 2002).

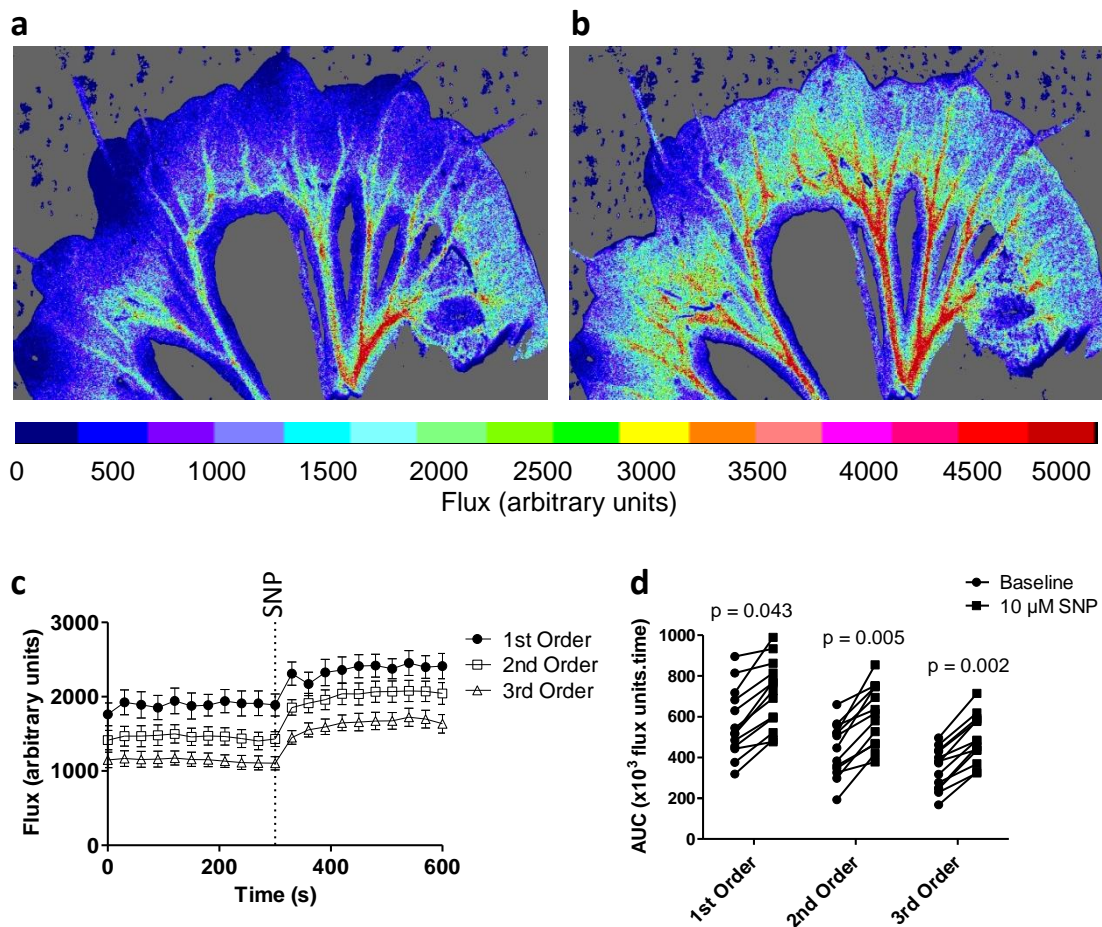


Figure 22. Effect of topical administration of vasodilator SNP on mesenteric blood flow in endotoxaemic mice (12.5 mg/kg LPS, i.v., 24 h). Representative flux images taken at baseline (**a**) and following topical administration of 10 μ M SNP (2 pumps of aerosolised spray; approx. 200 μ l; **b**) in endotoxaemic mice 24 h post-LPS. (**c**) Flux recorded over 5-min baseline and following topical administration of SNP, indicated by dotted line. Data are presented as mean \pm SEM (n = 12). (**d**) Area under the curve (AUC; $\times 10^3$ flux units.time) for each mouse over 5-min baseline recording and 5-min response to SNP. Measurements for each animal are denoted by individual symbols connected by a solid line (n = 12), and comparisons of baseline AUC with AUC post-SNP were assessed by Student's paired two-tailed T-test.

3.3.8 Bolus systemic administration of heparin does not improve mesenteric perfusion in sepsis

In order to elucidate the role of coagulation in sepsis-associated microvascular perturbations, a systemic bolus of heparin was administered at the time of sepsis induction. Mesenteric blood flow was assessed 24 h after the induction of sepsis or sham surgery. Heparin had no effect on mesenteric blood flow in LPS-treated mice (**Fig. 23a**). In CLP-treated mice, however, it appeared to exaggerate the decline in blood flow (**Fig. 23b**). Heparin was not observed to have any impact on arbitrary severity score, weight loss or core temperature (data not shown).

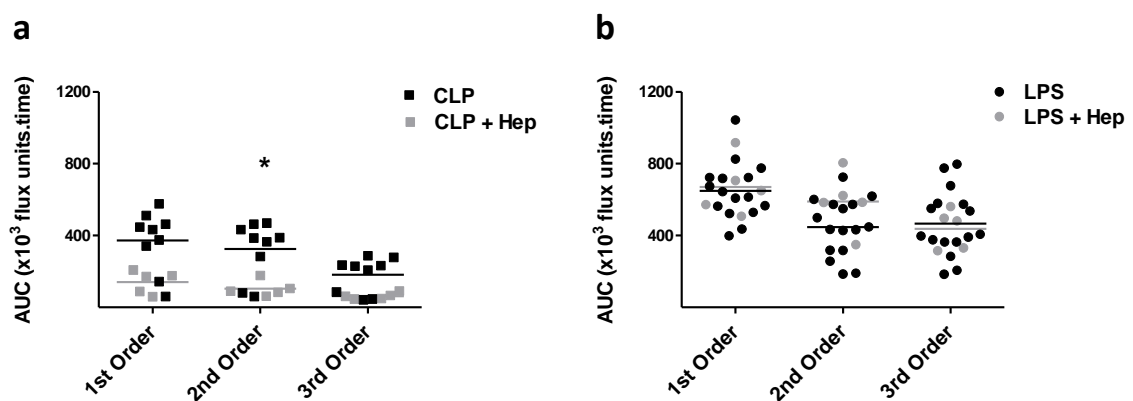


Figure 23. Impact of heparin on mesenteric blood flow in septic mice. Mesenteric blood flow expressed as total area under the curve (AUC; x10³ flux units.time) over 5-min baseline recording in 1st, 2nd and 3rd order mesenteric vessels in 24 h after the induction of sepsis by CLP (**a**) or LPS (**b**) either in the presence or absence of 100 U/kg heparin (Hep). Data for each animal are denoted by individual symbols, with horizontal bars denoting group mean (n = 5–12). Comparisons of control mice (black) and mice treated with heparin (grey) were performed in each vessel type. *p<0.05, 1-way ANOVA + Bonferroni post-hoc test.

3.3.9 Wider applications of the model

In order to assess other potential applications of the model, we used the ferric chloride model of acute thrombosis. Topical application of FeCl₃ (but not saline) induced a steady decline in blood flow over 30 min, resulting eventually in almost complete occlusion of the vessel. Representative images are shown in **Figure 24**; FeCl₃ reproducibly stimulated thrombus formation in 6 mice.

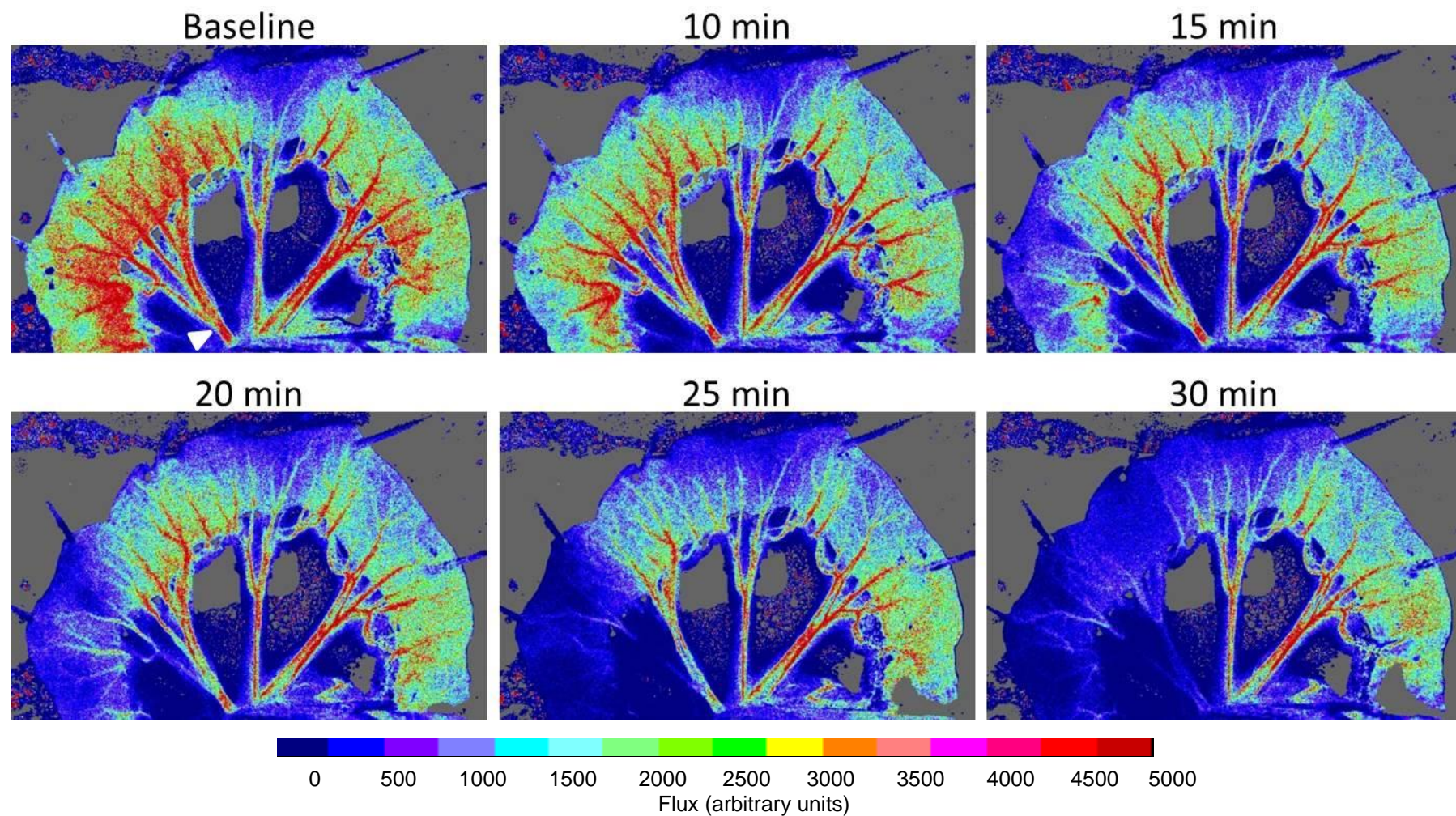


Figure 24. Ferric chloride model of acute thrombosis. Representative images of mesenteric blood flow following topical administration of a 1-mm² square of filter paper soaked in 20% FeCl₃ (indicated by white arrow head). Cold colours indicate low flow, warmer colours higher flow. Almost complete occlusion of the affected vessel was achieved within 30 min.

3.4 Summary

The key findings from this study are:

1. Laser speckle contrast imaging can be used in concert with other standard haemodynamic techniques to robustly assess microcirculatory perfusion in septic mice.
2. Microcirculatory flow, as quantified in the mesenteric and ear vascular beds, diminishes in a time-dependent manner in both models of sepsis.
3. Microcirculatory flow more closely correlates with end organ perfusion and metabolic disturbances than global haemodynamics.
4. Stabilisation of macrocirculatory haemodynamic parameters is likely to occur at the expense of microcirculatory perfusion, and is therefore insufficiently predictive of outcome. By extension, strategies aimed at elevating MAP pharmacologically may adversely affect microcirculatory perfusion.
5. Aggressive fluid resuscitation measurably improves microcirculatory perfusion and end organ function, consistent with clinical observations.
6. Topical administration of a vasodilatory agent can reverse sepsis-induced microcirculatory impairment, supporting the theory that compliant vascular beds are actively shut down in sepsis.
7. Systemic administration of the anticoagulant heparin had no effect on mesenteric perfusion in the LPS model, but exacerbates microcirculatory disturbances in the CLP model of sepsis.
8. The model described represents a novel approach to microcirculatory monitoring that can be used in concert with haemodynamic and haematological assessment. It is robust, reproducible and clinically relevant; moreover its use is not restricted to sepsis research: while we anticipate that its incorporation into pre-clinical sepsis models will facilitate more sophisticated and clinically relevant proof-of-concept studies, it may also be applied in *in situ* studies of vasoactivity or thrombosis.

3.5 Discussion

3.5.1 Refinement of pre-clinical sepsis models

A strong research drive and extensive media coverage have served to bring sepsis into public prominence in recent years. The failure of the PROWESS-SHOCK trial (Ranieri et al., 2012) and the withdrawal of activated Drotrecogin alfa – the only approved anti-sepsis drug – in 2011, have prompted much ‘soul-searching’ amongst critical care specialists and pre-clinical research scientists (Dolgin, 2012). A significant factor in the lack of effective treatments for septic shock, in addition to the limitations of related clinical trials (Khamsi, 2012), is the problem of research translatability; numerous interventions that have proved beneficial in animal models have failed to translate into improved outcome in humans (Raven, 2012). Patient and pathogen heterogeneity, and the confounding effects of clinical intervention complicate the process of developing a relevant model in which to investigate disease mechanisms. Pre-clinical focus on endpoints with low prognostic value, however, may also have contributed significantly to this issue. While research has tended to centre on global haemodynamics and crude mortality endpoints, models which focus on the mechanism of death, specifically perfusion failure, may be more clinically relevant. The aim of this study, therefore, was to investigate changes in microcirculatory blood flow in sepsis and its relation to haemodynamic and haematological indices, with a view to characterising the prognostic sensitivity of this parameter.

Because murine models facilitate gene modification, they currently represent the only viable option for conducting mechanistic pre-clinical proof-of-concept research. Therefore, it is of paramount importance to optimise the systems available to us by limiting confounding influences, and to employ animal models to their full capacity, extracting as much information as possible from a single individual. With this in mind, we aimed to establish a robust model for assessing microcirculatory perfusion in a septic mouse, and to incorporate this model into a multi-parameter monitoring system for direct comparison (in the same animal) of microvascular and global haemodynamic parameters, as presented schematically in **Figure 14**. The method described represents a novel application of laser speckle contrast imaging technology, allowing simultaneous and real-time visualisation of blood flow across entire sections of the mesenteric bed *in situ*, with no dissection of perivascular tissue, and minimal mechanical disruption to the vessels. This represents a significant advantage over traditional laser Doppler techniques that are limited to measurements in single vessels, often requiring extensive microdissection and probe-tissue contact, as well as conferring significantly lower processing speeds. Additionally, this technique does not require the use of fluorescent dyes, necessary for intravital microscopy, and furthermore, allows automated quantification of flow within specified regions of interest, both at baseline and following topical or intravenous

administration of a pharmacological agent. As such, this model represents a practical substitute to the sidestream dark field imaging technique that is used clinically, as it does not require high-level operating expertise, and is not subject to inter-operator variability or probe-tissue contact artefacts.

Both core temperature and mesenteric blood flow remained stable throughout the recording period, and for up to 60 min, validating the use of the model for assessing acute changes in vasoactivity in response to interventions, independent of changes in temperature or haemodynamic status. The presence of a telemetry probe in the flank of the mouse had no bearing on the flow values recorded.

We have demonstrated that this model is stable and reproducible, validating its use for assessment of acute vasoactive responses to intervention; it is also compatible with gold-standard techniques for cardiovascular evaluation, including radiotelemetry and echocardiography. Importantly, we have demonstrated that mesenteric blood flow diminishes in line with the development of metabolic acidosis and organ failure in sepsis, and that this is attenuated by an established clinical intervention, suggesting that our model represents a more clinically relevant and prognostic endpoint for pre-clinical sepsis research.

3.5.2 Mesenteric blood flow declines in a time-dependent manner in sepsis, and may be actively shut down to preserve global haemodynamics

Mesenteric blood flow declined in a time-dependent manner in both models of sepsis. LPS-treated mice exhibited a hypothermic phenotype at 6 h that was further exaggerated by 24 h; CLP-treated mice became gradually, but less severely hypothermic over 24 h, consistent with previous reports (Clark et al., 2007, Coldewey et al., 2013, Saito et al., 2003). Interestingly, whereas CLP induced less severe hypothermia than LPS, mesenteric blood flow was actually compromised to a greater extent in the former model, suggesting that microvascular impairment is the result of cardiovascular pathology, rather than an incidental effect of decreased core temperature.

While disseminated intravascular coagulation, vessel remodelling and hypovolaemia may all be expected to contribute to the observed changes in microvascular blood flow, our data suggest an important role for active vasoconstriction and shunting of blood away from the mesenteric bed during sepsis. It is especially noteworthy that in both models, sepsis-induced hypotension, and LPS-induced bradycardia stabilised after 9 h, with MAP values at 24 h equivalent to those at 6 h post-sepsis, despite a continued decline in mesenteric perfusion. Similarly, the decrease in cardiac output observed in both models by 6 h post-sepsis did not decline significantly thereafter. This suggests that systemic stabilisation may be achieved at the expense of the

microcirculation, as proposed previously (Spronk et al., 2004). Correspondingly, whilst CLP-treated mice appear to have less severe haemodynamic dysfunction than LPS-treated mice, microcirculatory function appeared further diminished in the former. While MAP in CLP-treated mice was not significantly different from baseline at 6 h post-sepsis, a marked decrease in mesenteric perfusion was observed at this time point, as exemplified in **Figure 19**.

While acute arterio-venous shunting may be essential to preserve global circulation and perfusion of heart and brain tissue in acute hypovolaemia, prolonged microcirculatory shut-down, most notably in the splanchnic region, inevitably leads to hypoxic tissue injury and the development of organ failure (Takala, 1996, Spronk et al., 2004). Clinically described as ‘the motor of multiple organ failure’ (Carrico et al., 1986), the gut represents an important microcirculatory bed in septic shock, where impaired flow is known to correlate strongly with poor outcome both in animal models (Baykal et al., 2000) and septic patients. (Takala, 1997, Spronk et al., 2004). This likely reflects the fact that impaired perfusion of the intestine, and associated hyperpermeability, facilitates the leakage of microorganisms and endotoxin from the gut lumen into the lymphatic and cardiovascular circulation, exacerbating the inflammatory state (Sautner et al., 1998). The observation that sepsis-induced microvascular impairment can be reversed by the application of a topical vasodilator further supports the theory that compliant vascular beds are actively shut down in sepsis in order to preserve global circulation. Circulatory bypassing of microvascular beds could also explain why venous oxygen saturation (SO₂) was did not decrease in either model of sepsis.

Although beyond the scope of this study, future research in which an arterial catheter is used to measure blood pressure in parallel with blood flow measurements could shed further light on the clinical benefits of dilating regional vascular beds. If peripheral vasodilatation (in the gut, for example) can be achieved without compromising arterial pressure beyond the threshold required for global oxygen delivery, this may represent a potential therapeutic strategy in the treatment of sepsis.

Administration of norepinephrine – known to raise arterial pressure – far from increasing regional perfusion actually diminished mesenteric blood flow, suggesting firstly that prolonged vasopressor use may accelerate organ failure, and secondly that treatment strategies targeted at global haemodynamics may not be effective, as demonstrated previously (Gattinoni et al., 1995). The theory that increasing global oxygen delivery will necessarily increase regional delivery seems overly simplistic, and due consideration must be given to the regional pharmacological activity of specific vasopressors; noradrenaline-mediated constriction of small vessels may exacerbate regional ischaemia, for example. While other vasopressors, such as

dobutamine, may have less severe effects on microcirculatory blood flow (Hernandez et al., 2013), these data highlight the importance of monitoring loco-regional perfusion directly, especially when administering vasopressor therapy.

Our data emphasise that stabilisation of macrocirculatory parameters may not indicate halting of syndrome progression, and therefore should not be used as a prognostic marker in isolation. This conclusion is supported by clinical evidence: numerous trials of interventions that improve hemodynamic performance in animal models (Ullrich et al., 2000, Connelly et al., 2005) and humans, have failed to confer a survival benefit in clinical trials (Kirov et al., 2001, Lopez et al., 2004, Gutierrez et al., 1992). We have demonstrated that direct measurement of mesenteric perfusion – known to confer high prognostic value in the clinic (Gutierrez et al., 1992, Ivatury et al., 1996, Palizas et al., 2009) – is not only feasible in a murine model, it is also more sensitive to syndrome progression.

3.5.3 Microcirculatory shut-down may become maladaptive and precipitate metabolic disturbance and organ failure

While mesenteric blood flow did not appear to mirror changes in global haemodynamic status, it did reflect time-dependent changes in metabolic disturbance and organ dysfunction: blood pH decreased and blood urea levels increased in a time-dependent manner in line with diminished perfusion.

Both blood pH and bicarbonate levels were decreased in both models of sepsis, indicative of metabolic acidosis. Consistent with compensation for metabolic acidosis, partial pressure of CO₂ (PCO₂) was found to decrease at 6 h in both models, presumably as a result of tachypnea – a common feature of sepsis, and a criterion for diagnosis (Bone et al., 1992a) that results in early respiratory alkalosis. The increased workload associated with tachypnea can lead to fatigue and eventual failure of the respiratory muscles, and diversion of blood flow towards the respiratory muscles and away from other vital organs can result in organ injury (Kellum, 2007). By 24 h, PCO₂ levels had increased relative to the 6-h time point in both models, suggesting failure of compensatory mechanisms. In the CLP model, PCO₂ levels had increased above baseline levels (albeit non-significantly), indicative of respiratory acidosis (Kellum, 2004). The raised anion gap, particularly in the LPS model, is also indicative of acid accumulation.

Metabolic acidosis may arise by a number of different mechanisms, including hyperchloraemia, lactic acidosis, and renal failure (Kellum, 2004). Hyperchloraemia can occur in sepsis if bicarbonate is lost through the GI tract or kidneys, resulting in chloride retention; equally, elevated chloride levels may also be the result of saline resuscitation (administered at the time of sepsis). Both lactic acidosis and renal failure are consequences of poor organ

perfusion and insufficient oxygen delivery. While lactate was only measured in the fluid resuscitation experiments, non-resuscitated CLP-treated mice exhibited elevated lactate levels relative to sham controls. Furthermore, the time-dependent rise in blood urea levels observed in both models of sepsis is indicative of impaired renal perfusion and kidney dysfunction (Rosner and Bolton, 2006).

Surprisingly, given that sepsis is associated with plasma extravasation, which tends to induce haemoconcentration, both haematocrit and haemoglobin levels were found to decrease following the induction of sepsis. One possible explanation for this phenomenon is breakdown of the endothelial glycocalyx, a carbohydrate-rich layer overlying the vascular endothelium, previously shown to be degraded in sepsis (Steppan et al., 2011, van den Berg et al., 2003). Shedding of this layer, estimated to be 0.2 to 0.5 μm thick (van den Berg et al., 2003), may lead to an increase in intravascular space, and consequently to haemodilution. A decrease in haemoglobin levels has also been observed in septic patients (Jansma, 2013). These observations, and ours, may be linked with reports of hyporesponsiveness to erythropoietin (EPO) in sepsis (Nissenon et al., 2006), which would limit the production of erythrocytes by bone marrow. Acute kidney injury, frequently encountered in sepsis (Zarjou and Agarwal, 2011), may also impair EPO production by interstitial kidney fibroblasts. Haemolysis may also be a factor in the sepsis-associated haemodilution observed here: fibrin strands generated as a result of disseminated intravascular coagulation can destroy erythrocytes (Bull and Kuhn, 1970); the complement system, which is also activated in sepsis, has also been associated with haemolysis (Adamzik et al., 2012), as has LPS itself (Poschl et al., 2003). Sepsis has also been associated with eryptosis, or the suicidal death of erythrocytes (Lang et al., 2010).

Overall, a good agreement between mesenteric perfusion and markers of acid-base disturbance and organ dysfunction was observed, suggesting that microcirculatory blood flow is a more sensitive marker of syndrome progression than haemodynamic indices.

3.5.4 Microcirculatory blood flow is a clinically relevant endpoint in pre-clinical sepsis models

In order to determine the clinical relevance of the mesenteric microcirculation model, we tested its ability to reflect improved outcome in response to fluid resuscitation – an intervention that has been shown definitively to increase survival in patients (Rivers et al., 2001). In the more clinically relevant CLP model, a resuscitation protocol was employed based on one known to attenuate mortality in septic mice (Zanotti-Cavazzoni et al., 2009). This intervention modestly, but significantly, improved mesenteric perfusion, as well as a number of other clinical parameters: fluid-resuscitated mice also exhibited modest improvements in

hypothermia, arbitrary severity score (assigned in a blinded fashion), metabolic acidosis, and urea and lactate production. Interestingly, measurement of perfusion by the same method in a different microvascular bed – the ear – revealed a significant decrease in blood flow in CLP-treated mice that was reversed by fluid resuscitation. Measurement of blood flow in the ear may represent a less invasive method for repeated-measures testing of microcirculatory function over a specific time-course, given that it does not require terminal anaesthesia, although further validation of this endpoint is required.

Whether the observed improvement in microcirculatory perfusion was simply the result of improved global haemodynamic performance is uncertain; MAP and cardiac output (although not measured in this study) would undoubtedly have increased with fluid loading. Our own experiments with noradrenaline, however, as well as numerous other studies (Dubin et al., 2009, Jhanji et al., 2009), have demonstrated that raising MAP does not increase regional perfusion *per se*. It is likely, therefore, that correction of hypovolaemia played a more important role in the microvascular changes observed here. Indeed, although macrohaemodynamics and microcirculatory flow are both affected by hypovolaemia, and tend to improve in parallel with fluid resuscitation, their relative changes are not well correlated, and fluid resuscitation is thought to have direct beneficial effects on the microcirculation in patients (Hernandez et al., 2012b).

Irrespective of the mechanism involved, these data suggest that the mesenteric model is clinically relevant and may be adapted to a comparatively hyperdynamic murine model of sepsis. The extent to which a mouse can reflect the truly hyperdynamic state that occurs in continuously infused patients is undoubtedly severely limited by its size. Murine models may be used effectively to understand the pathophysiological changes that occur when sepsis is allowed to progress unimpeded, however, and to establish the impact of pharmacological or genetic modification in the absence of other interventions. To mimic more closely the resuscitated intensive care situation, larger species should be used, where administration of large volumes of fluid in addition to pharmacological agents is more feasible.

3.5.5 Bolus systemic administration of heparin does not improve mesenteric blood flow in murine models of sepsis

Disseminated intravascular coagulation is a common feature of sepsis and septic shock (Semeraro et al., 2012), and microvascular thrombus formation is likely to contribute to regional ischaemia and organ dysfunction. An anti-coagulant – heparin – was administered at the time of sepsis, in order to determine the impact on mesenteric blood flow. Surprisingly, heparin had no effect on blood flow in the LPS model. Although heparin acts immediately

following intravenous administration, its half-life in plasma is approximately 40–90 minutes (Rang et al., 2007); while evidence of coagulopathy is already present at 6 h post-sepsis in mice (Corral et al., 2005), it is possible that by 24 h after LPS protective effects had worn off, with pro-coagulant systems predominating. In the CLP model, heparin-treated mice exhibited an exaggerated impairment in blood flow relative to untreated controls. In this model, initial transient coagulation may help to contain bacteria within the septic focus, facilitating destruction by immune cells; the early actions of heparin in this case, may have accelerated the systemic dissemination of bacteria, exacerbating the inflammatory response.

Whether the mesenteric flow impairments recorded here are truly independent of coagulation (and predominantly the result of active shunting), or whether our experimental design was unsuited to the detection of measurable changes following anti-coagulant administration remains unclear. Unfortunately, continuous infusion of heparin over a 24-h period, which may have prevented coagulopathy in later stages, was not feasible in these models. Furthermore, while repeated bolus dosing may have been beneficial, the dissection required for the experimental set-up may have induced excessive bleeding and produced artefactual results. It is equally possible that later administration of a thrombolytic agent, rather than an anti-coagulant, could have reversed the impaired mesenteric flow observed in both models. Nonetheless, the failure of anti-coagulant therapies to improve survival in clinical trials (Freeman et al., 2003), combined with their associated bleeding complications, suggests that the coagulation cascade may not represent a fruitful avenue of research for potential sepsis treatments.

3.5.6 Use of murine models in sepsis research

Recent reports of divergent genomic responses to inflammation between mice and men (Seok et al., 2013), compounded by associated media furore, have called into question the validity of mouse models of inflammatory conditions, including sepsis. Early diagnosis and intervention are certainly key to limiting sepsis-associated mortality (Brown et al., 2013, Bravi et al., 2012), and investigation of diagnostic biomarkers must necessarily be conducted in humans, as described recently (Ma et al., 2013b). Calls for murine models of inflammation to be abandoned entirely, however, are certainly injudicious. As the only species in which genetic modification is both feasible and reliable, the mouse retains enormous value in delineating the involvement of particular proteins in sepsis pathogenesis, and physiological responses to inflammation are largely conserved between species. Furthermore, much of the recent criticism of murine studies of inflammation (Seok et al., 2013) has itself been flawed: it is doubtful that meaningful conclusions may be drawn from a comparison of the leukocyte gene

expression profiles of a heterogeneous, co-morbid population of patients, with a homogeneous cohort of age-matched and genetically similar mice, as argued recently (Osuchowski et al., 2014).

Because animal models currently represent the only viable option for conducting pre-clinical research, it is critical that we optimise the systems available to us by limiting confounding influences, and that we employ animal models to their full capacity, extracting as much information as possible from a single individual transitioning from health to disease. Importantly, the cardiovascular consequences of sepsis observed in this study – hypotension, microcirculatory impairment, metabolic acidosis – are in line with clinical observations, validating the mouse model for investigation of physiological processes in sepsis.

This study has demonstrated a novel comprehensive approach to modelling sepsis pathogenesis, which encompasses multi-parameter monitoring in individual animals. We have shown that a number of biological measurements can be performed in a single mouse without compromising data quality or reproducibility, and without incurring undue suffering. While results, in sepsis research in particular, should always be interpreted with caution, and never over-extrapolated, proof-of-concept studies in well-designed mouse models with clinically relevant endpoints can provide valuable information on the likely impact of a particular intervention on a physiological system, and can inform the design of relevant clinical trials. This is particularly important in the later stages of sepsis, where mortality rates are unacceptably high, and where there is a strong requirement for adjunct therapies.

3.5.7 Concluding remarks

To our knowledge, this study represents the first comprehensive multi-parameter assessment at all levels of the cardiovascular system in a single mouse. Mice were found to exhibit both haemodynamic disruption and microvascular impairment following the induction of sepsis, though the latter correlated more closely with markers of organ dysfunction and metabolic acidosis.

The comprehensive multi-parameter model developed in this study and shown schematically in **Figure 14**, confers a number of significant advantages to previous methods employed in pre-clinical sepsis research. Firstly, all monitoring is undertaken remotely or under anaesthetic, eliminating the risk of stress artefacts associated with blood pressure measurements obtained through tethering or tail cuff recording. Secondly, it enables *in situ* monitoring of all levels of the cardiovascular system – cardiac, macrohemodynamic, microcirculatory and haematological – in a single animal, from the naïve state through disease progression. This study has

demonstrated the feasibility of conducting a paired study that mimics perioperative recording of the transition from health to disease. This type of analysis provides a much more comprehensive assessment of health, and, as demonstrated, can be achieved without mutual interference. Furthermore, the mesenteric model is not limited to sepsis research, and can also be applied to *in situ* studies of acute thrombogenesis/thrombolysis or vasoactive responses to systemic or topical stimuli, negating the need for dissection and organ bath preparations, which can lead to tissue damage artefacts. Finally, use of this model is in keeping with the remit of the National Centre for the Replacement, Refinement and Reduction (NC3Rs) of Animals in Research: multi-parameter monitoring reduces the number of mice required for a study, and use of a more clinically relevant and prognostic endpoint represents a refinement of pre-clinical modelling.

In summary, this study has demonstrated that microcirculatory perfusion is severely compromised in sepsis, in a manner which correlates closely with end organ damage (the ultimate cause of death in septic patients); results indicate that macrocirculatory parameters alone may have limited prognostic value. The author believes that quantification of microcirculatory blood flow, as described, provides a humane pre-mortality endpoint, whilst simultaneously improving the clinical translatability of pre-clinical sepsis studies. Information gained using this comprehensive and clinically relevant method of monitoring sepsis parameters, with particular focus on microcirculatory function, will help to refine the development of novel interventions, and limit attrition in later stages.

CHAPTER 4 | THE ROLE OF TRPV1 IN SEPSIS-ASSOCIATED MICROVASCULAR DYSFUNCTION

4.1 Introduction

4.1.1 Properties of TRPV1

Transient receptor potential vanilloid 1 (TRPV1), originally termed vanilloid receptor type 1 (VR1), was identified by Caterina et al. in 1997 and characterised as a sensor of noxious heat ($> 42^{\circ}\text{C}$) (Caterina et al., 1997). It is additionally and uniquely activated by capsaicin (Caterina et al., 1997), the active ingredient in chilli peppers, as well as other structurally related vanilloids (such as resiniferatoxin; RTX) (Caterina et al., 1997), protons (Tominaga et al., 1998), and endovanilloids, including anandamide and arachidonic acid-derived lipoxygenase products (Ross, 2003, Holzer, 2008). Activation of TRPV1 is competitively antagonised by capsazepine, a synthetic analogue of capsaicin, though since its initial characterisation (Bevan et al., 1992, Urban and Dray, 1991) several potent and more selective TRPV1 antagonists have been developed. Widely expressed, and implicated in a wide range of physiological processes, the receptor has received much research interest since its initial characterisation. It is predominantly studied in the contexts of nociception and thermosensation, though wider roles for TRPV1, particularly in the cardiovascular system, are emerging.

4.1.1.1 Structure, function and expression of TRPV1

TRPV1 subunits have large intracellular N- and C-termini, with a short hydrophobic pore-forming region spanning transmembrane domains 5 and 6 (**Fig. 25**) (Caterina et al., 1997). The N-terminus contains multiple ankyrin repeat domains, essential for channel function, and proposed to bind cytosolic proteins, including calmodulin (Lishko et al., 2007). The C-terminus contains a TRP domain, suggested to function as a molecular determinant of subunit tetramerisation (Garcia-Sanz et al., 2004), which, in the case of TRPV1, is predominantly homomeric (Kedei et al., 2001). Also in the C-terminus is a binding site for membrane lipid phosphatidylinositol-4,5-bisphosphate (PIP_2), which can both positively and negatively regulate channel activity, depending on its positioning in the upper or lower leaflet of the cell membrane (Senning et al., 2014).

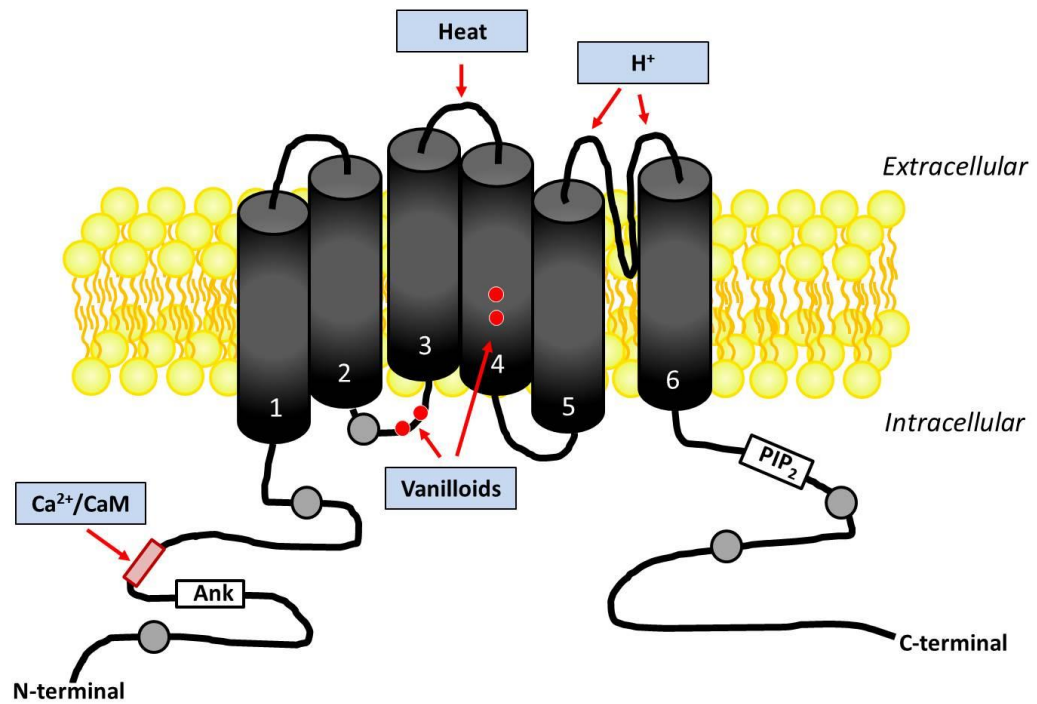


Figure 25. Structural features of the TRPV1 subunit. TRPV1 subunits comprise 6 transmembrane domains with a re-entrant pore-forming loop between domains 5 and 6, and cytosolic N- and C-termini. The N-terminus contains three ankyrin (Ank) repeat domains and a Ca²⁺-calmodulin (CaM) binding site. The C-terminal contains a binding site for phosphatidylinositol 4,5-bisphosphate (PIP₂). Grey circles represent potential phosphorylation sites for protein kinases A and C; red circles represent binding sites for vanilloids, including capsaicin. Heat- and acid-sensing residues are located on extracellular loops.

Consistent with the observation that intradermal injection of capsaicin causes intense pain in humans and pain-related behaviour in experimental animals (Baumann et al., 1991), TRPV1 is predominantly localised in nociceptive C and A δ afferents (Caterina et al., 1997). These fibres [typically small- to medium-diameter neurons projecting from dorsal root, trigeminal and nodose sensory ganglia (Caterina et al., 1997)] characteristically contain neuropeptides, such as calcitonin gene-related peptide (CGRP), substance P (SP), neurokinin A and somatostatin (Alawi and Keeble, 2010). Nociceptor occupation triggers non-specific cation influx and neuron depolarisation, which in turn induces an afferent impulse conduction and pain sensation, as well as neuropeptide release (Holzer, 1988). Na⁺ influx is sufficient to mediate the former response, whereas Ca²⁺ influx, and thus extracellular Ca²⁺, is required for neuropeptide release (Holzer, 2008).

In addition to its neuronal expression, there is increasing evidence of TRPV1 expression in non-neuronal tissues, including keratinocytes (Southall et al., 2003), urothelial cells (Birder et al., 2001), vascular smooth muscle (Ma et al., 2011), endothelium (Golech et al., 2004), and inflammatory cells, such as lymphocytes (Saunders et al., 2007) and macrophages (Chen et al., 2003, Fernandes et al., 2012), as well as a number of brain regions (Mezey et al., 2000). Its functions and indeed its very presence in these tissues, however, remain a topic of intense debate and form the subject of much current investigation.

4.1.1.2 Receptor desensitisation

The response of TRPV1 to capsaicin exhibits profound and Ca²⁺-dependent desensitisation by two distinct and independent mechanisms: prolonged capsaicin exposure induces ‘acute desensitisation’ involving a calmodulin-dependent inactivation of the ionic current, whereas repeated application of capsaicin causes ‘tachyphylaxis’, or a reduction in the maximal current amplitude (Koplas et al., 1997) likely due to receptor internalisation and degradation (Sanz-Salvador et al., 2012). In addition to causing desensitisation or endocytosis of the channel itself, capsaicin is also known to cause neurotoxicity and defunctionalisation following repeated application to nerve terminals both *in vivo* (Jancso et al., 1977) and *in vitro* (Wood et al., 1988). This necrosis is thought to occur through ionic overload and mitochondrial dysfunction, and is specifically TRPV1-dependent (Caterina et al., 1997). Indeed, experimental denervation by capsaicin has emerged as a highly useful tool for investigating the importance of TRPV1 and sensory nerve input in numerous physiological systems.

4.1.2 TRPV1 and cardiovascular regulation

The efferent-like function of capsaicin-sensitive sensory nerve fibres was first described in the 1960s, with several reports of stimulus-induced release of locally-acting neuropeptides from peripheral nerve terminals (Holzer, 1988). TRPV1-positive sensory nerves project predominantly to cardiovascular and renal tissues, and are found in close proximity with blood vessels in almost all vascular beds (Wang, 2005). A significant proportion of these afferents contain the highly vasoactive neuropeptides CGRP and SP, which are synthesised in the dorsal root ganglia (DRG) that contain the cell bodies of capsaicin-sensitive sensory neurons (Wang, 2005). Local release of CGRP (Brain et al., 1985) and SP (Maggi, 1995) forms a key component of neurogenic vasodilatation.

The contribution of neurogenic vasodilatation to blood pressure regulation (through modification of peripheral resistance vessels) was first suggested in 1988 (Kawasaki et al., 1988). This seminal study described a non-adrenergic, non-cholinergic vasodilatory response to peripheral nerve stimulation, which was proposed to contribute to the regulation of vascular tone in mesenteric resistance beds, and was abolished following depletion of CGRP from perivascular nerve endings (Kawasaki et al., 1988). The role of TRPV1-positive sensory nerve fibres in blood pressure regulation has since been the subject of intense investigation, and recent studies have shown that sensory nerves interact with and counterbalance several pro-hypertensive neurohormonal systems [including the renin-angiotensin-aldosterone system (Wang and Li, 1999), the sympathetic nervous system (Wang et al., 2001) and the endothelin system (Ye and Wang, 2002)] to maintain normal blood pressure. More recently, however, deletion of TRPV1 has been associated with exaggerated hypertension induced by high fat feeding, perhaps as a result of exaggerated vascular remodelling (Marshall et al., 2013).

Interestingly, while capsaicin has generally been reported to cause vasorelaxation, there have also been reports of TRPV1-mediated vasoconstriction in mesenteric (Scotland et al., 2004), coronary (Szolcsanyi et al., 2001) and skeletal muscle (Kark *et al.*, 2008) arteries, and in the mouse knee joint (Keeble and Brain, 2006). Scotland et al. (2004) described a contractile response to both increased intraluminal pressure and TRPV1 activation in small mesenteric resistance arteries that was sensitive to antagonists capsazepine and ruthenium red and was abolished following sensory denervation with a single intraperitoneal injection of 50 mg/kg capsaicin (Scotland et al., 2004). This response was found to be dependent on

activation of vascular smooth muscle neurokinin 1 (NK₁) receptors by SP, and was suggested by the authors to contribute to myogenic tone regulation.

It is unclear, however, how or why endogenous activation of TRPV1 in this context causes preferential release of SP over CGRP (a potent vasodilator) (Brain et al., 1985) or indeed why the SP released should act on vascular smooth muscle NK₁ receptors to cause vasoconstriction, rather than endothelial NK₁ receptors to cause vasodilatation, as is more commonly observed (Maggi, 1995, Lembeck and Holzer, 1979, Gamse et al., 1980, Gazelius et al., 1981).

One possible explanation is that the response reported by Scotland et al. (Scotland et al., 2004) is regionally restricted, and that the role of TRPV1 in vascular regulation may vary according to the type and localisation of circulatory system (Inoue et al., 2008). Alternatively, in light of reports of a direct vasoconstrictor effect of capsaicin on vascular smooth muscle (Holzer, 1991, Szolcsanyi et al., 2001, Kark et al., 2008), the possibility that myogenic constriction is mediated through a non-neuronal mechanism warrants further elucidation. Given that the authors did not consider the effect of intraperitoneal capsaicin on non-neuronal TRPV1, which may also be desensitised through such treatment, it is possible that the myogenic constriction observed may indeed have been mediated by direct effects of TRPV1 activation in vascular smooth muscle cells. This would certainly be consistent with previous publications indicating no direct role for periarterial nerves in the vascular myogenic response (Bayliss, 1902, Johansson, 1989, Marshall et al., 2013).

In addition to its activity in perivascular nerves, TRPV1 may also contribute to vascular function directly. There have been numerous reports of TRPV1 expression in vascular endothelium (Golech et al., 2004, Yang et al., 2010a, Bratz et al., 2008) and smooth muscle tissue (Cavanaugh et al., 2011, Czikora et al., 2012, Kark et al., 2008), where it is thought to contribute particularly to microvascular regulation.

4.1.3 TRPV1 as a key player in the relationship between infection, inflammation and cardiovascular dysfunction

4.1.3.1 Neurogenic inflammation

The release of neuropeptides from sensory nerve terminals following TRPV1 activation has long been recognised to play an important role in the inflammatory milieu. CGRP and SP, in particular, are considered pro-inflammatory mediators, eliciting increased blood flow (Brain et al., 1985) and plasma extravasation (Lembeck and Holzer, 1979), respectively, characteristic of neurogenic inflammation. Activation of NK₁ receptors by SP has also been implicated in microvascular leukocyte recruitment under inflammatory conditions (Cao et al., 2000, Costa et al., 2006), and in the upregulation of interleukin-8 (IL-8) – a chemoattractant and potent proangiogenic factor (Zhao et al., 2002). These processes appear to be highly significant in the pathogenesis of a number of conditions, including rheumatoid arthritis, colitis, asthma and migraine (Holzer, 2008).

Release of somatostatin, on the other hand, is associated with an anti-inflammatory effect, which involves an auto-inhibitory mechanism in which it acts on sensory nerve terminals to prevent further neuropeptide release (Helyes et al., 2007, Pinter et al., 2006). In addition to its prejunctional effects, somatostatin also acts postjunctionally: binding of somatostatin to G-protein coupled receptors on endothelial, inflammatory and immune cells inhibits the action of pro-inflammatory neuropeptides at their effector sites (Pinter et al., 2006).

4.1.3.2 Direct stimulation of pro-inflammatory cytokine production

In addition to mediating neurogenic inflammation, TRPV1 may also be functional in inflammatory cells themselves. Receptor expression has been detected in human leukocytes (Spinsanti et al., 2008) and mast cells (Stander et al., 2004), and Biro et al. have demonstrated TRPV1-induced production of pro-inflammatory cytokine IL-4 in murine mast cells (Biro et al., 1998). In humans, Stander et al. (2004) observed sporadic mast cell expression of TRPV1 under basal conditions, though in skin samples from patients with mastocytosis (an inflammatory skin condition) almost all mast cells showed TRPV1 immunoreactivity, which was confirmed by qRT-PCR and Western blot analysis (Stander et al., 2004). These data were interpreted as indicative of an 'autocrine loop of receptor upregulation' that may stimulate a pro-inflammatory state through increased cytokine production.

Interestingly, despite historically having been considered a pro-inflammatory receptor, a number of recent studies suggest that TRPV1 may confer protective effects in some inflammatory settings (Alawi and Keeble, 2010).

4.1.3.3 Evidence of a protective role for TRPV1 in sepsis

The importance of sensory nerve input in sepsis was first inferred from two observations: first, the exacerbated inflammatory responses to infection following sensory denervation of experimental animals (Bowden et al., 1996); and second, the raised plasma levels of neuropeptides CGRP and SP in septic patients (Joyce et al., 1990, Beer et al., 2002). More recently, an upregulation of TRPV1 and CGRP in tongue tissue and mesenteric perivascular nerves, respectively, has been demonstrated in endotoxaemic rats (Orliac et al., 2007), suggesting that changes in TRPV1 and neuropeptide expression may contribute to the physiological responses to sepsis.

Indeed, a role for TRPV1 in mediating a fever response to endotoxin has been demonstrated both in sensory denervated rats (Romanovsky, 2004) and TRPV1 KO mice (Iida et al., 2005). Interestingly, and perhaps counterintuitively, TRPV1 has also been implicated in protection against sepsis-induced cardiovascular dysfunction. Wang et al. demonstrated that recovery from endotoxin-induced hypotension and tachycardia in anaesthetised mice was strongly inhibited by both capsazepine and selective NK₁ receptor antagonism (Wang et al., 2008a). 24- and 48-h survival of conscious rats treated with lipopolysaccharide (LPS) was similarly improved, suggesting that TRPV1 may mediate cardiovascular protection in this setting via the release of SP. In support of this, plasma levels of SP were significantly raised in LPS-treated rats, compared with vehicle-treated controls (Wang et al., 2008a).

Further investigation in our laboratory has revealed that TRPV1 KO mice exhibit significantly enhanced hypotension compared to WT counterparts at 4 h post-LPS administration, with early signs of liver dysfunction, including significant liver plasma extravasation and raised levels of plasma aspartate aminotransferase, apparent in LPS-treated TRPV1 KO but not WT mice (Clark et al., 2007). However, given that induction of cytokines, such as IL-6, only occurs 6 h after the induction of endotoxaemia in mice, and haematological changes become pronounced after 24 h (Xiao et al., 2006), it seems likely that a 4-h end point is too early to fully assess the functional effects of TRPV1 deletion in a murine model of sepsis. Furthermore, the possibility of developmental compensation for TRPV1 gene ablation

cannot be ruled out. Selective and tissue-specific TRPV1 antagonism *in vivo* may be helpful in elucidating this further.

A more recent study, using the cecal ligation and puncture (CLP) model of sepsis found that at 24 h post-CLP, TRPV1 KO mice exhibit enhanced hypotension compared to WT controls, and plasma markers of heart, liver, kidney and pancreas dysfunction were significantly raised in the former group (Fernandes *et al.*, 2012). Decreased mononuclear cell integrity and enhanced peritoneal apoptosis were observed in the septic focus of KO mice, as well as decreased macrophage NK₁-dependent phagocytosis. Moreover, isolated macrophages from TRPV1 KO mice exhibited an impaired ability to phagocytose latex beads *in vitro*, following stimulation with LPS. This process in WT mice was found to be dependent on SP (Fernandes *et al.*, 2012). These data suggest that deletion of the TRPV1 gene leads to impaired bacterial clearance in sepsis, accelerating the transition from local infection to a full systemic inflammatory response syndrome, and the onset of organ damage and failure.

In contrast with reports described above, a recent study by Wang and Wang found no difference in haemodynamic performance between LPS-treated TRPV1 WT and KO mice. Immunohistochemical and blood marker analysis, in contrast, revealed markedly exaggerated organ dysfunction and tissue neutrophil recruitment in the latter (Wang and Wang, 2013). These data are consistent with results outlined in **Chapter 3**, showing that arterial pressure is insufficiently indicative of syndrome progression. A possible reason for this discrepancy in results obtained by Wang and Wang compared with previous investigations is the nature of haemodynamic monitoring techniques employed: while reports of exaggerated hypotension in LPS-treated TRPV1 KO mice have used either invasive catheterisation under anaesthesia (Wang *et al.*, 2008a) or the tail-cuff method (Clark *et al.*, 2007, Fernandes *et al.*, 2012), known to induce significant stress artefacts, Wang and Wang used radiotelemetry in unrestrained conscious mice – known to represent the gold standard in haemodynamic monitoring (Morton *et al.*, 2003). Nonetheless, while the receptor may not play a significant role in haemodynamic regulation in sepsis, TRPV1-mediated protection against inflammation-induced organ damage is now well established.

The endogenous agonist for this protective response has yet to be identified, although the endocannabinoid anandamide is a potential candidate, as several studies have reported increased anandamide production by macrophages and endothelial cells during endotoxaemia (Batkai *et al.*, 2004, Varga *et al.*, 1998). Although activation of cannabinoid receptor CB1 by anandamide elicits profound hypotension (Varga *et al.*, 1996), it has been

demonstrated *ex vivo* that the vasodilatory response to anandamide in rat mesenteric arteries occurs predominantly through TRPV1 (Zygmunt et al., 1999) and this is supported by *in vivo* studies demonstrating that the cardiac depressor response to methanandamide is attenuated by capsazepine (Wang et al., 2005).

Thus far, the longer-term effects of TRPV1 deletion on vascular function in sepsis have not yet been investigated. It is possible that, in addition to immune cell regulation, TRPV1 may also mediate vasoactive effects: given its ability to cause both vasoconstriction and vasodilatation, depending on its cellular localisation, TRPV1 may contribute to recovery from septic hypotension through the former mechanism, or the maintenance of organ perfusion through the latter. This may form a basis for understanding why septic TRPV1 KO mice appear to exhibit both exaggerated hypotension and accelerated organ dysfunction (Clark et al., 2007), though this requires further investigation.

4.1.4 Hypotheses

Previous findings within our laboratory as well as others suggest that TRPV1 is protective in the setting of sepsis. Given the growing body of evidence establishing TRPV1 as a potential regulator of vascular function – particularly in small resistance arteries, where dysfunction correlates strongly with adverse outcome in sepsis – we hypothesise that TRPV1 may mediate protective effects through its ability to regulate vascular function in inflammatory conditions.

4.1.5 Aims

The aims of this study were as follows:

1. To use a novel model of microcirculatory assessment, described in **Chapter 3**, to determine the impact of TRPV1 deletion on microcirculatory blood flow
2. To relate any changes in blood flow to other measurements of disease severity, including blood markers of acidosis and organ dysfunction, and other macroscopic clinical parameters
3. To determine the impact of topical TRPV1 agonism on mesenteric blood flow in healthy and septic wildtype mice

4.2 Methods

4.2.1 Animals

Male wildtype (WT) and TRPV1 knockout (KO) mice were bred in house from heterozygous crosses, as described in section 2.1. Mice were used between 10 and 14 weeks of age (approx. 25–30g).

4.2.2 Induction of sepsis

Experimental sepsis was induced by intravenous injection of lipopolysaccharide (LPS; 12.5 mg/kg) from *Salmonella typhimurium* (Sigma, L7261). Injections of approximately 100 µl (pre-warmed to 37°C) were administered into the tail vein under inhaled isoflurane anaesthesia, as described in section 2.3.3.1. Mice were placed in a recovery chamber at 27°C for up to 24 h and were monitored frequently. An arbitrary severity score of 1–5 (based on mobility, facial expression, piloerection and aversion to touch) was employed to assess animal welfare. Any mouse reaching a score of 5 was immediately terminated.

Given its ability to recapitulate the cardiovascular alterations associated with sepsis (as demonstrated in **Chapter 3**), the LPS model was chosen based on its practicality, and to prevent any confounding influence of divergent responses to surgery between genotypes.

4.2.3 *In situ* monitoring of mesenteric blood flow

Mesenteric blood flow was measured in naïve and septic mice 24 h post-LPS, as described in **Chapter 3**; a rectal probe coupled to a homeothermic heating mat was used to maintain mice at the temperature at which they presented. After 5 min baseline recording, the mesenteric bed was gently sprayed (2 pump compressions from a distance of approximately 10 cm) with either 10 µM capsaicin dissolved in saline containing 1% DMSO or vehicle, pre-warmed to 37°C. This concentration, approximately 10-fold higher than the EC₅₀ for TRPV1 in heterologous expression systems (Caterina et al., 1997), was chosen based on the aerosolised and relatively diffuse delivery mode. Responsiveness to capsaicin was measured over a further 5 min; a venous blood sample was subsequently drawn from the inferior vena cava, and animals were terminated by cervical dislocation. Blood biochemistry was assessed immediately from 100 µl venous blood using a hand-held iSTAT® point-of-care analyser (Abbott Laboratories, IL, USA), with CG8+ cartridges (Abbott Laboratories). Results were analysed in moorFLPI Review software (V4.0) and GraphPad Prism, as described in section 3.2.7.1. Results are expressed as mean area under the curve (AUC) over time ± SEM.

4.3 Results

4.3.1 TRPV1 KO mice are homeostatically stable during blood flow recording

TRPV1 KO mice, like WT mice, exhibited stable core temperature (Fig. 26d) and mesenteric blood flow (Fig. 26a–c) during blood flow recording, validating the use of these animals in studies of vasoactive responses to intervention that are not confounded by changes in temperature or haemodynamic status. In this series of experiments, 3 animals were excluded from the LPS group, and 2 from the naïve group, due to bleeding during dissection for blood flow recording.

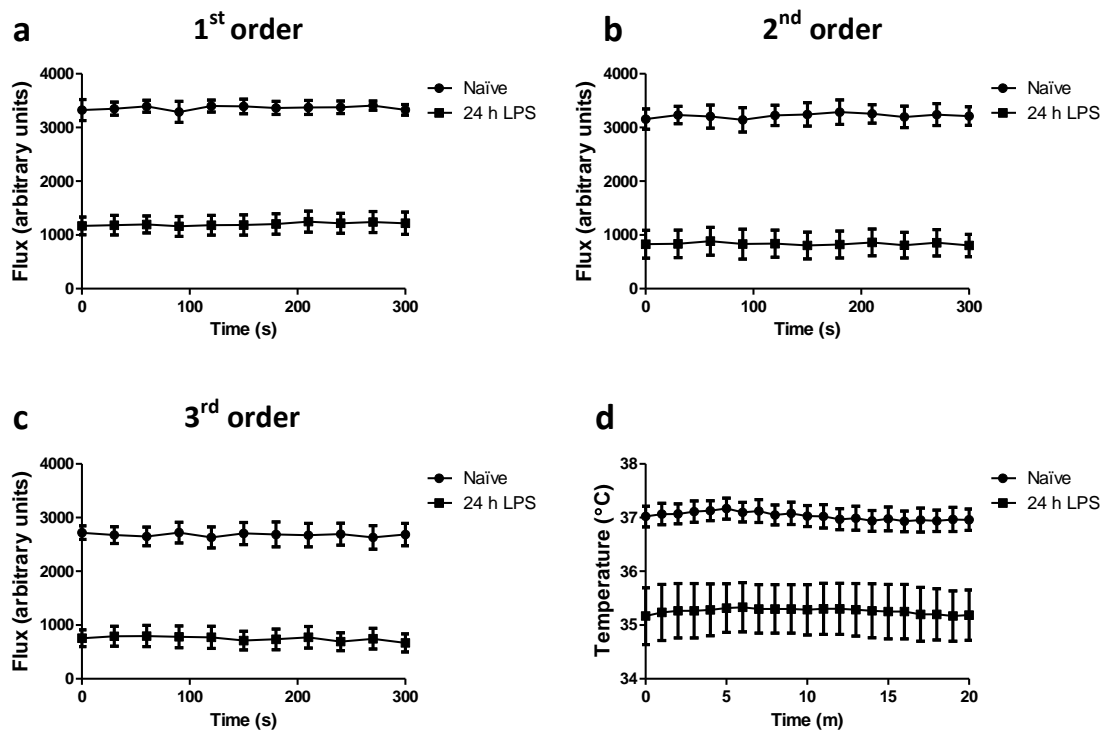


Figure 26. Mesenteric blood flow and core temperature over time in naïve and septic TRPV1 KO mice. Blood flow in **(a)** 1st, **(b)** 2nd and **(c)** 3rd order mesenteric vessels was stable over the recording period. Core temperature **(d)** was also stable over the recording period, suggesting that vasoactive responses recorded are not influenced by changes in temperature or haemodynamic status. Data are presented as mean \pm SEM ($n = 7-9$).

4.3.2 TRPV1 KO mice have exaggerated mesenteric flow impairment in LPS-induced sepsis

Mesenteric blood flow was equivalent between TRPV1 WT and KO mice under naïve conditions, though there was a slight trend for decreased flow in 1st and 3rd order vessels in the latter. Following the induction of sepsis by intravenous injection of LPS, however, the flow impairment in KO animals was exaggerated relative to WT controls (**Fig. 27**), especially in smaller 3rd order vessels (**Fig. 27c**) that directly supply gut tissue, where flow was significantly decreased relative to LPS-treated WT controls.

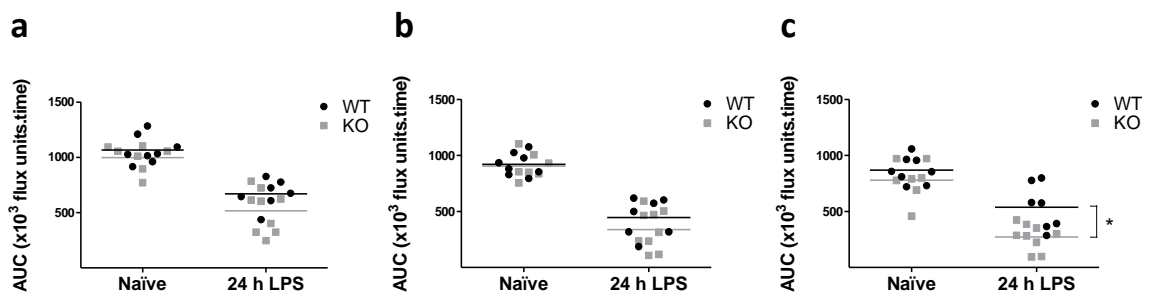


Figure 27. Mesenteric blood flow in TRPV1 WT and KO mice under naïve and septic conditions. Mesenteric blood flow is expressed as total area under the curve (AUC; x10³ flux units.time) over 5-min baseline recording in (a) 1st, (b) 2nd and (c) 3rd order vessels. AUC for each animal is represented as an individual symbol, with horizontal line denoting group mean. *p<0.05, 1-way ANOVA + Bonferroni post-hoc test (n = 7–9).

4.3.3 Deletion of TRPV1 is associated with a worse clinical phenotype in LPS-induced sepsis

TRPV1 KO mice exhibited an exaggerated phenotype in LPS-induced sepsis by a number of additional parameters. Arbitrary severity score (assigned in a blinded fashion based on voluntary mobility, gait, aversion to touch, facial expression and piloerection) was significantly increased in KO animals (**Fig. 28a**; $p = 0.04$, student's unpaired two-tailed T-test). Hypothermia during sepsis was slightly exaggerated in KO animals (**Fig. 28b**), although weight loss over the 24-h sepsis time course was equivalent between genotypes (**Fig. 28c**). Initial body weight (prior to the induction of sepsis) was slightly lower in KO mice, however (**Fig. 28d**), despite a similar change over the sepsis period (mean $\Delta g \pm SEM$: 2.75 ± 0.32 vs 2.78 ± 0.19 in WT and KO mice, respectively, ns). TRPV1 KO mice also had a slightly higher core temperature than WT mice under naïve conditions ($37.03 \pm 0.24^\circ\text{C}$ vs 36.75 ± 0.22) though this was not statistically significant. Mice were age-matched between genotypes.

Blood from LPS-treated TRPV1 KO mice was slightly more acidotic than WT controls, and urea was also slightly higher, though these changes were not statistically significant. PCO_2 was also elevated in LPS-treated KOs relative to WTs, bicarbonate levels were slightly reduced, and base excess and anion gap were slightly exaggerated. Interestingly, naïve KO mice also exhibited trends towards basally pathological blood biochemistry. Urea was slightly elevated in these mice, and pH slightly decreased, relative to WT controls. Bicarbonate levels were also slightly decreased in KO mice, and basal glucose levels were higher. These data are summarised in **Table 8**.

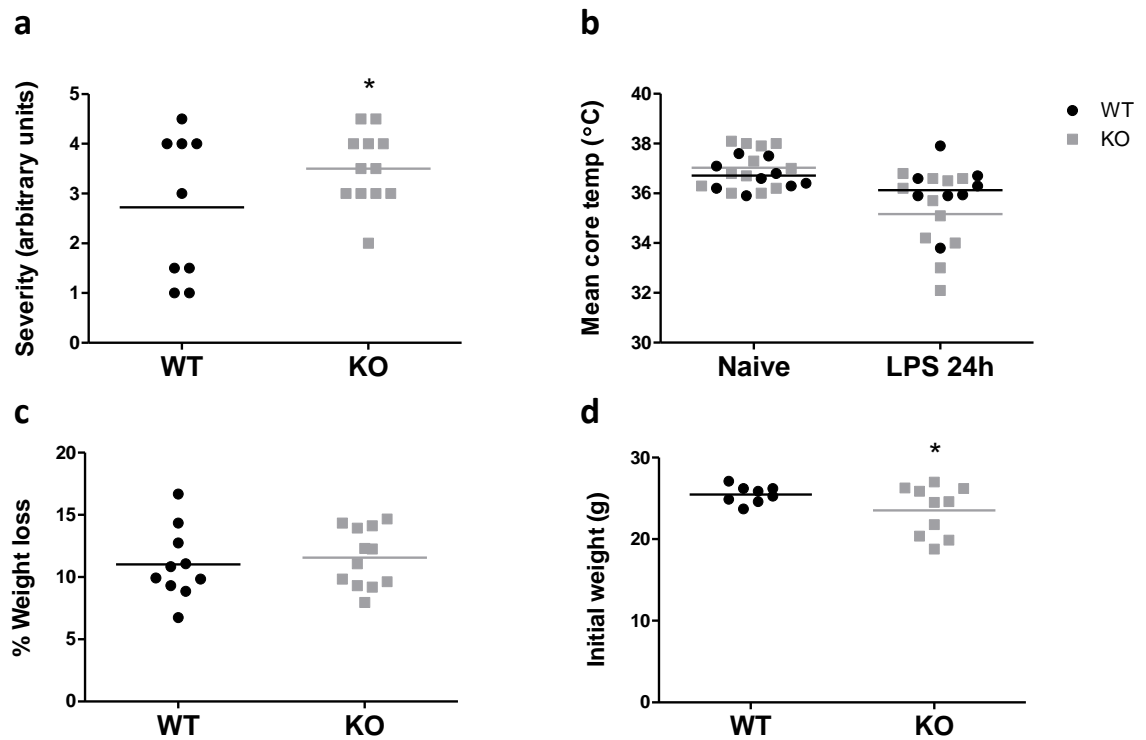


Figure 28. Clinical parameters in TRPV1 WT and KO mice. (a) Arbitrary severity score assigned in a blinded fashion based voluntary mobility, gait, aversion to touch, facial expression and piloerection. (b) Initial temperature taken immediately after induction of anaesthesia for blood flow recording. (c) Percentage weight loss over 24-h septic period (d) Initial body weight prior to the induction of sepsis, taken 24 h before blood flow recording. Values for each animal are presented as individual symbols, with horizontal line denoting group mean. * $p < 0.05$, Student's unpaired two-tailed T-test ($n = 9-12$).

	WT Naïve	KO Naïve	WT LPS 24 h	KO LPS 24 h
Urea (mmol/L)	4.74 ± 0.63	5.91 ± 0.65	33.62 ± 5.56***	39.61 ± 4.074***
pH	7.227 ± 0.04	7.186 ± 0.044	7.005 ± 0.07*	6.845 ± 0.05***
Base excess (mmol/L)	-10.89 ± 0.99	-12.86 ± 2.19	-20.67 ± 2.35**	-25.00 ± 1.50***
HCO₃⁻ (mmol/L)	16.86 ± 0.73	10.42 ± 1.35 ^{##}	15.41 ± 1.40	8.43 ± 0.95 ^{##}
PCO₂ (mmHg)	41.62 ± 3.18	40.96 ± 3.92	40.10 ± 2.63	48.01 ± 5.09
TCO₂ (mmol/L)	18.25 ± 1.44	16.86 ± 1.37	11.67 ± 1.36*	10.00 ± 1.03**
Glucose (mmol/L)	11.03 ± 0.66	14.89 ± 1.80	2.33 ± 0.31**	1.87 ± 0.19***
Na⁺ (mmol/L)	150.3 ± 0.25	148.8 ± 0.68	152.2 ± 0.79	155.3 ± 2.80
K⁺ (mmol/L)	3.425 ± 0.43	4.133 ± 0.26	3.583 ± 0.29	4.089 ± 0.66
Cl⁻ (mmol/L)	109.0 ± 2.08	108.5 ± 1.13	117.3 ± 1.11*	115.8 ± 1.77**
Anion gap (mmol/L)	27.33 ± 3.28	26.17 ± 2.01	28.25 ± 2.36	37.75 ± 8.79
Haematocrit (%PCV)	35.50 ± 2.18	35.00 ± 1.82	30.33 ± 1.99	28.11 ± 2.50
Haemoglobin (g/dl)	12.39 ± 0.75	11.79 ± 0.80	10.17 ± 0.75	9.58 ± 0.85

Table 8. Blood biochemistry in healthy and septic TRPV1 KO and WT mice. Blood gas and biochemistry were measured from venous blood samples by iSTAT point-of-care analyser in naïve septic mice at 24 h post-LPS. Data are presented as mean ± SEM. *p<0.05, **p<0.01 and ***p<0.001 relative to respective naïve controls; ^{##}p<0.01 relative to respective WT controls, 1-way ANOVA and Bonferroni post-hoc test, n = 7–11.

Abbreviations: PCO₂, partial pressure of CO₂; TCO₂, total carbon dioxide; SO₂, venous oxygen saturation; PCV, packed cell volume.

4.3.4 Vasoactive responses to topical capsaicin administration may be altered in endotoxaemic WT mice

In order to assess changes in TRPV1 activity *in vivo* after the induction of sepsis, 10 μ M capsaicin or vehicle (2% DMSO in pre-warmed saline) was applied onto the exposed mesentery of WT mice by aerosolised spray. In naïve mice, topical administration of capsaicin caused a decrease in blood flow in 1st order vessels (**Figs. 29a & 30a**). While vehicle also caused a small decrease in blood flow, this was significantly greater following stimulation with capsaicin (mean percentage change \pm SEM: $-2.19 \pm 2.62\%$ vs $-13.95 \pm 2.56\%$, $p < 0.01$, 1-way ANOVA + Bonferroni post-hoc test). In 2nd order vessels, vehicle and capsaicin caused a decrease in blood flow of a similar magnitude (mean percentage change \pm SEM: $-7.89 \pm 1.78\%$ vs $-8.02 \pm 1.57\%$, respectively, ns; **Figs. 29b & 30b**), suggesting the effect was not mediated by TRPV1. In 3rd order vessels, vehicle slightly decreased blood flow, whereas capsaicin caused a small increase (mean percentage decrease \pm SEM: $-2.65 \pm 1.15\%$ vs $+3.69 \pm 1.74$, ns; **Figs. 29c & 30c**).

In LPS-treated mice, on the other hand, capsaicin caused a small increase in blood flow in all vessels, though vehicle produced a similar effect (albeit of a smaller magnitude). In 1st order vessels mean percentage change \pm SEM was $2.70 \pm 1.93\%$ vs $14.52 \pm 2.67\%$ for vehicle and capsaicin, respectively (ns; **Figs. 29d & 30d**). In 2nd order vessels mean percentage change \pm SEM was $7.40 \pm 1.85\%$ vs $20.37 \pm 6.92\%$ for vehicle and capsaicin, respectively (ns; **Figs. 29e & 30e**). In 3rd order vessels mean percentage change \pm SEM was $-3.11 \pm 1.48\%$ vs $21.51 \pm 5.89\%$ for vehicle and capsaicin, respectively ($p < 0.01$, 1-way ANOVA + Bonferroni post-hoc test; **Figs. 29f & 30f**).

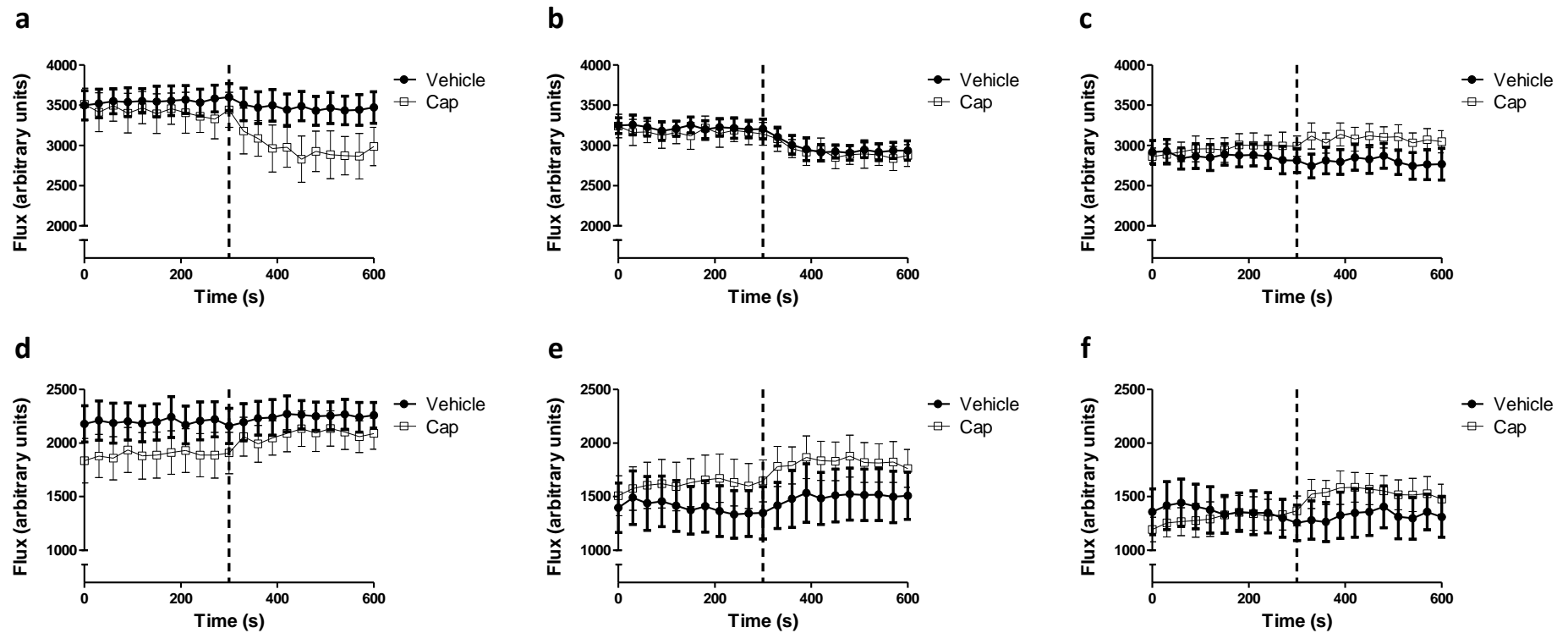


Figure 29. Vasoactive responses to capsaicin and vehicle in healthy and septic WT mice. Baseline mesenteric blood flow was recorded for 5 min in naïve (**a–c**) and septic (**d–f**) mice, 24 h after injections of LPS. Capsaicin (Cap; 10 μ M) or vehicle (2% DMSO in saline) was then administered as an aerosolised spray, denoted by the dotted line, and blood flow was recorded for a further 5 min. In naïve mice, capsaicin caused a decrease in blood flow in (**a**) 1st order and (**b**) 2nd order vessels (though vehicle also decreased flow in the latter) but not in (**c**) 3rd order vessels. In LPS-treated mice, however, capsaicin increased blood flow in (**d**) 1st, (**e**) 2nd and (**f**) 3rd order vessels. Data are presented as mean \pm SEM, n = 6–14.

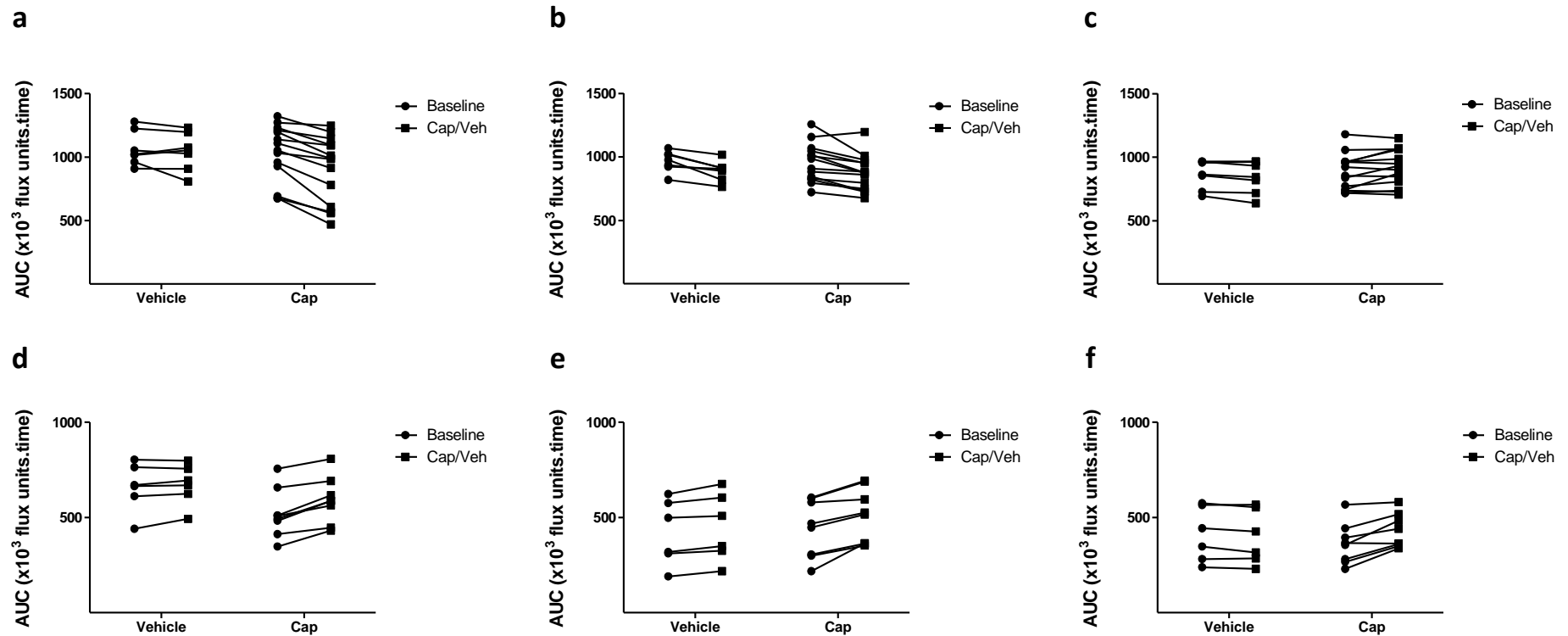


Figure 30. Mesenteric blood flow at baseline and following topical administration of capsaicin or vehicle in healthy and septic mice. Area under the curve (AUC; flux units.time) over 5-min baseline recording and 5-min response to topical administration of capsaicin (Cap; 10 μ M) or vehicle (2% DMSO in saline) are denoted by closed black circles and open white squares respectively. In naïve mice, AUC decreased in **(a)** 1st order and **(b)** 2nd order vessels (though vehicle also decreased flow in the latter) but not in **(c)** 3rd order vessels. In septic mice (24 h LPS), however, capsaicin increased AUC in **(d)** 1st, **(e)** 2nd, and **(f)** 3rd order vessels. AUC for each animal is denoted by individual symbols connected by a black line (n = 6–14).

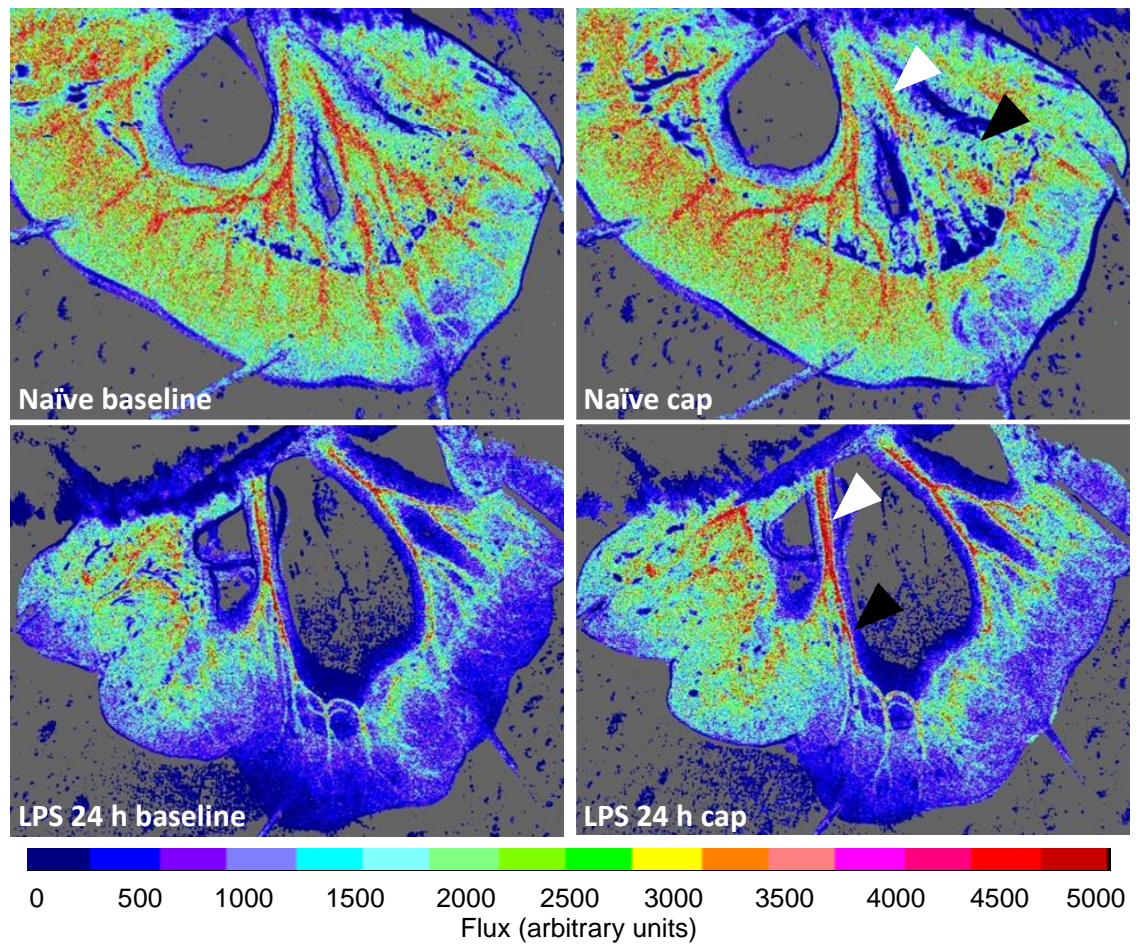


Figure 31. Representative flux images baseline and following topical administration of capsaicin in healthy and septic mice. Cold colours indicate low flow; warm colours, high flow. Topical administration of capsaicin (cap; 10 μ M) by aerosolised spray caused a small decrease in flow in 1st and 2nd order vessels of naïve mice; and a small increase in blood flow in all vessels of septic mice (24 h LPS). White arrowheads indicate 1st order vessels; black arrowheads indicate 2nd order vessels.

4.4 Summary

The key findings from this study are:

1. TRPV1 KO mice, like WT mice, are homeothermically and haemodynamically stable under the conditions used for blood flow monitoring.
2. Naïve TRPV1 KO mice exhibited a trend towards basal acidosis.
3. TRPV1 KO mice exhibit exaggerated microvascular flow impairment and clinical pathology in LPS-induced sepsis.
4. Vasoactive responses to topical administration of capsaicin may be altered in LPS-induced sepsis.

4.5 Discussion

Vascular dysfunction, and consequent perfusion impairment, is a major contributor to sepsis-associated mortality. Numerous reports of a protective role for TRPV1 in sepsis have been published in recent years (Wang et al., 2008a, Clark et al., 2007, Fernandes et al., 2012); in light of the growing body of evidence establishing TRPV1 as a potential regulator of vascular function – particularly in small resistance arteries, where dysfunction correlates strongly with adverse outcome in sepsis – we hypothesised that TRPV1 may mediate protective effects through its ability to regulate vascular function in inflammatory conditions. We aimed to compare the cardiovascular phenotype of TRPV1 WT and KO mice under naïve and septic conditions using our novel model of microcirculatory assessment, described in **Chapter 3**. We additionally aimed to relate any changes in blood flow to other measurements of disease severity, including blood markers of acidosis and organ dysfunction, as well as a variety of macroscopic clinical parameters. Finally we aimed to determine the impact of topical TRPV1 agonism on mesenteric blood flow in healthy and septic wildtype mice.

4.5.1 TRPV1 KO mice exhibit a trend towards basal acidosis

Initially characterised by Caterina et al., TRPV1 KO mice are reported to be largely indistinguishable from WT littermates in terms of general appearance, gross anatomy, body weight, locomotion and overt behaviour (Caterina et al., 2000). Conversely, our TRPV1 KO mice were slightly but significantly leaner than their WT littermates, consistent with other reports that TRPV1 KO mice are leaner when young but have a greater tendency towards obesity with age (Moran et al., 2011). Furthermore, analysis of blood biochemistry in naïve KO mice revealed evidence of slight basal metabolic acidosis. Whereas naïve WT mice had a mean blood pH of 7.227 – normal for C57/BL6 mice (Tordoff et al., 2007) – naïve TRPV1 KO mice had a mean blood pH of 7.186 and significantly lower blood bicarbonate levels with normal PCO₂, consistent with metabolic acidosis (Lien and Lai, 1998). These observations are consistent with reports that TRPV1 is the main nociceptive acid-sensor in mice (Leffler et al., 2006).

Furthermore, while basal blood glucose levels in WT mice were consistent with previous reports (Berglund et al., 2008), they were slightly elevated in TRPV1 KO mice. The reason for this observation is not clear, though it may relate to recent reports of a hypermetabolic phenotype in TRPV1 KO mice (Riera et al., 2014), which could also explain the slightly lower body weight associated with TRPV1 deletion observed here. Hypermetabolism is also associated with hyperglycaemia (Buczkowska, 2002), which may account for the slightly elevated blood glucose levels recorded in TRPV1 KO mice. Since mice were not fasted prior to

the measurement of blood glucose, however, and since food intake was not monitored in this study, whether these differences are due to increased intake or metabolic factors is unclear.

4.5.2 TRPV1 KO mice exhibit exaggerated pathogenesis in LPS-induced sepsis

While microvascular blood flow in naïve mice was equivalent between genotypes, perfusion was reduced to a greater extent in septic TRPV1 KO mice, reaching statistical significance in the smallest 3rd order vessels. This may account for the accelerated organ dysfunction and increased mortality in septic mice lacking a functional TRPV1 receptor reported previously (Wang et al., 2008a, Clark et al., 2007, Fernandes et al., 2012), and is consistent with previous reports that TRPV1 KO mice have a greater tendency towards peripheral vasoconstriction (Garami et al., 2011).

In line with reduced mesenteric perfusion, septic TRPV1 KO mice also exhibited enhanced pathogenesis by a number of other clinical parameters. Blood levels of urea and markers of metabolic acidosis were elevated in the KO mice relative to WT littermates, and LPS-induced hypothermia was exaggerated in the former. It is noteworthy that this cohort of WT mice did not exhibit significant hypothermia, however, whereas previous cohorts exhibited a significant drop in core temperature at 24 h post-LPS (**Chapter 3; Fig. 5**). Indeed in previous cohorts, mean temperature at 24 h post-LPS was $33.9 \pm 0.71^{\circ}\text{C}$ – markedly lower than the mean temperature ($35.87 \pm 0.44^{\circ}\text{C}$) recorded in this cohort. Whereas an initial period of hypothermia following intravenous administration of LPS is well-documented, numerous studies have reported a return to baseline temperatures by 24 h (Souza et al., 2014, Saia and Carnio, 2006), consistent with measurements for WT mice in this cohort. While the differences in hypothermic responses measured between cohorts in this study may be explained by fluctuations in ambient temperature (Rudaya et al., 2005), or by batch differences in LPS, WT and KO littermates in this study were studied under identical conditions, suggesting that the changes in hypothermic response are real.

Overall the data recorded here are consistent with a protective role for TRPV1 in sepsis. There are numerous caveats to this conclusion, however. Firstly, given the propensity of genetic ablation to induce compensatory upregulation of similarly functioning genes during development, it would be necessary to repeat these studies using selective antagonists of TRPV1. Furthermore, the role of TRPV1 in inflammatory conditions appears to become more complicated with increasing age. Whereas selective antagonism of TRPV1 in 12-week old mice enhances LPS-induced mortality (consistent with the present data), both pharmacological antagonism and genetic deletion of TRPV1 delay and decrease mortality in 43-week old LPS-treated mice (Wanner et al., 2012). While TRPV1 is suggested to suppress production of

tumour necrosis factor (TNF) α in young endotoxaemic mice, this anti-inflammatory effect appears to be reversed in older mice, where suppression of TRPV1 activity is associated with decreased serum TNF α levels (Wanner et al., 2012). On the other hand, deletion of TRPV1 in aged mice subjected to cecal ligation and puncture was associated with exaggerated pathology (Wanner et al., 2012), which may indicate suppression of anti-microbial defence systems, as suggested previously (Fernandes et al., 2012). A more extensive time-course, in which the effects of TRPV1 deletion on sepsis progression are monitored at different time points, and for longer periods, would also be useful in elucidating this further.

Overall, research suggests that in the context of systemic inflammation, TRPV1 may evolve from an anti-inflammatory to a pro-inflammatory receptor with increasing age, whereas its role in the setting of infection may be more constant. Unfortunately, it was not possible to monitor changes in mesenteric blood flow in TRPV1 KO mice subjected to CLP in this study, owing to time constraints, though it would be interesting to see whether microvascular flow is similarly compromised in this model.

4.5.3 Vasoactive responses to topical capsaicin administration may be altered in LPS-induced sepsis

In situ investigation of vasoactive responses to TRPV1 activation was carried out in order to test the hypothesis that previously observed protective functions of TRPV1 in animal models of endotoxaemia (Wang et al., 2008a, Clark et al., 2007) may involve a vasoregulatory component. We hypothesised that changes to TRPV1 expression and/or activity under inflammatory conditions may underlie its protective effects.

A topical method of application was chosen to assess vasoactivity directly, since systemic administration of capsaicin may be expected to cause reflex changes in blood pressure. Mesenteric vasoactivity has traditionally been studied by myographical methods, though this *ex vivo* approach is limited by a number of factors. Firstly, the relevance of an isolated perfused vessel to the complex physiological environment encountered *in situ* is uncertain, and furthermore, the mounting process itself can introduce mechanical disruption to the vessel, most notably endothelial damage and inappropriate tension. While other groups have established methods of monitoring mesenteric blood flow *in situ* in rodents, these techniques have typically required significant microdissection of perivascular fat and connective tissue, and are generally restricted to the measurement of blood flow in a single vessel (Pourageaud and De Mey, 1997, Rowland et al., 2012, Hinohara et al., 2010). The method employed here allows for simultaneous visualisation of blood flow in several different vessels, with no perivascular dissection, as well as *in situ* assessment of acute drug effects via an aerosolised

topical delivery method conferring minimal mechanical and refractory disturbance, as discussed more extensively in **Chapter 3**.

A relatively high concentration of capsaicin [10 μ M – approximately 10-fold higher than the EC₅₀ in heterologous expression systems (Caterina et al., 1997)] was chosen for topical administration owing to the relatively diffuse delivery method. In naïve animals, capsaicin caused a small decrease in blood flow in 1st order vessels, consistent with vasoconstriction. While vehicle also caused a slight decrease in blood flow, this was of a smaller magnitude, indicative of at least a partially TRPV1-dependent effect. The reason for the vehicle-induced decrease in blood flow (particularly evident in 2nd order vessels) is unclear, and may be explained by evaporative cooling or a DMSO-mediated effect; DMSO has traditionally been associated with increased blood flow (Haller et al., 1987), however, though it has also been associated with cytotoxicity, notably in endothelial cultures (Sperling and Larsen, 1979), which could contribute to vascular dysfunction and perfusion impairment. On the other hand, blood flow in 3rd order vessels was not reduced by either vehicle or capsaicin, suggesting that the constriction observed in 1st order vessels in particular, may have been due to a specific pharmacological action.

Because TRPV1 activation on sensory nerves and in endothelial cells is generally associated with vasodilatation, it is conceivable that capsaicin-induced vasoconstriction could be attributed to TRPV1 expressed in smooth muscle cells, where its activation may lead to calcium-induced vasoconstriction, either directly or through local SP release, as suggested previously (Scotland et al., 2004). This is supported by observations that vasoconstriction occurs predominantly in 1st order vessels, likely to contain a substantial smooth muscle layer, rather than in 3rd order vessels, which directly supply the gut wall and are unlikely to contain a high proportion of smooth muscle tissue. The possibility of functional TRPV1 expression in smooth muscle cells is supported by a number of previous reports of capsaicin-induced vasoconstriction, as well as some direct evidence of smooth muscle TRPV1 expression (Kark et al., 2008, Bratz et al., 2008, Yang et al., 2010b, Scotland et al., 2004, Cavanaugh et al., 2011, Ma et al., 2011), as discussed in section **4.1.2.3**.

If indeed TRPV1 is expressed in the smooth muscle cells of these vessels, it is possible that it contributes to the myogenic response to increased blood pressure, as suggested previously (Scotland et al., 2004). It should be noted, however, that the concentration of capsaicin used here was relatively high. While previous reports that TRPV1 knockout mice show no aversion to water supplemented with 100 μ M capsaicin (Caterina et al., 2000) suggest that higher concentrations of the compound remain specific for TRPV1, off-target effects cannot be ruled

out. Further investigation of vasoactive responses to capsaicin in TRPV1 KO mice would elucidate this further.

In endotoxaemic mice capsaicin caused a subtle but consistent vasodilatation in all orders of mesenteric vessel. The switch from vasoconstriction to vasodilatation in response to capsaicin administration in sepsis is intriguing. It is possible that in the murine mesenteric bed, the pro-dilatory drive mediated by perivascular nerves (and potentially endothelial TRPV1) competes with a pro-constrictive drive mediated by smooth muscle TRPV1, and that under basal conditions a net constriction occurs that is reversed in inflammation (**Fig. 32**). Increased production of anandamide, by circulating macrophages and endothelial cells themselves, for example, may contribute to greater TRPV1-mediated endothelium-dependent vasodilatation in sepsis. Experiments in which sensory nerves are ablated, or in which neurogenic vasodilatation is blocked by CGRP and SP antagonists, as well as *in vitro* assessment of smooth muscle cell responsiveness to increasing concentrations of capsaicin could shed further light on this hypothesis. It would also be useful to perform immunofluorescent analysis of mesenteric artery sections from naïve and septic mice, in order to determine the overlap of TRPV1 expression with markers of sensory nerve terminals, endothelial cells and smooth muscle cells.

Increasing levels of circulating NGF in the inflammatory milieu, as well as direct contact between endothelial cells and circulating cytokines and inflammatory mediators may lead to a selective upregulation of neuronal and/or endothelial TRPV1 in sepsis, sufficient to overcome the constrictive activity of smooth muscle TRPV1. Consistent with this hypothesis, increased plasma levels of SP and CGRP have been observed in both patients with sepsis and animal models of sepsis (Joyce et al., 1990, Beer et al., 2002), and increased TRPV1 and CGRP immunoreactivity has been observed in tongue tissue and mesenteric perivascular nerves, respectively, after 6 h of LPS treatment in rats (Orliac et al., 2007). It is possible therefore, that TRPV1-mediated vasodilatation is a protective mechanism in sepsis that helps to maintain tissue perfusion. Whether this is mediated through purely neuronal mechanisms, however, or whether endothelial TRPV1 is also involved, remains to be determined.

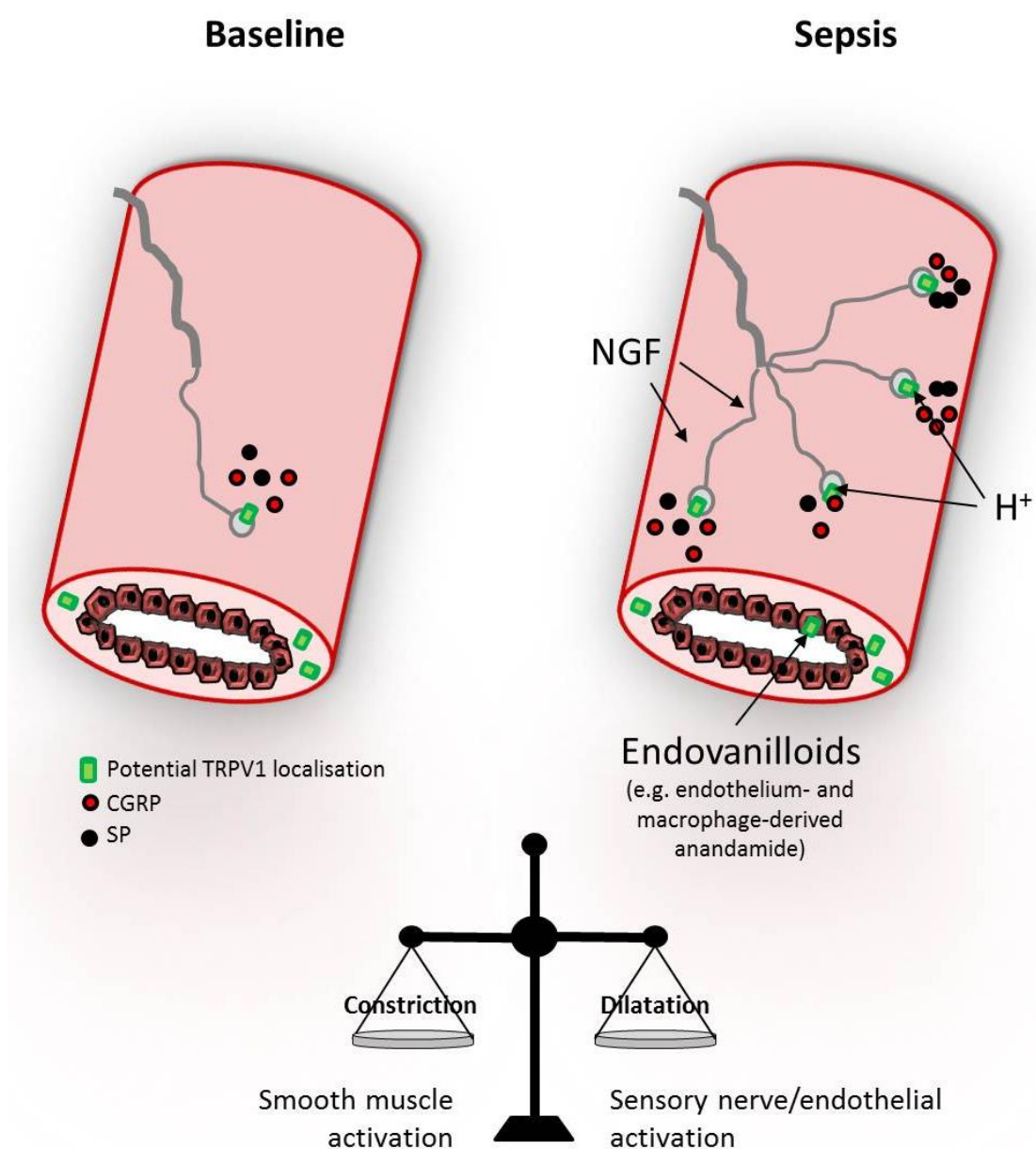


Figure 32. Proposed mechanisms of TRPV1-mediated vasoactive responses in the mesenteric bed under basal and septic conditions. Under basal conditions, smooth muscle-mediated constriction appears to dominate. Under inflammatory conditions, increased sensory nerve expression, previously shown to occur as early as 6 h after LPS administration in mice (Orliac et al., 2007), increased sensory nerve activation by protons, and increased endothelial activation through paracrine stimulation with anandamide may tip the balance in favour of TRPV1-mediated vasodilatation.

4.5.4 Concluding remarks

Overall the data obtained in this study are consistent with a protective role for TRPV1 in sepsis that may relate to local vasodilatation and the maintenance of regional perfusion. Of particular note is the observation, consistent with previous reports, that visceral perfusion is more severely compromised in LPS-treated TRPV1 KO mice, despite previous reports of equivalent haemodynamic performance between septic WT and KO mice (Wang and Wang, 2013). These data strongly support the results outlined in **Chapter 3**, showing poor correlation between haemodynamic indices and both microvascular perfusion and markers of organ dysfunction. Whether the regional protective effects of TRPV1 are mediated through neuronal mechanisms, however, or whether TRPV1 contributes to vasoregulation directly, remains unclear.

Irrespective of the mechanisms involved, the age-dependent effects of TRPV1 activity, as well as its divergent roles in different models of sepsis (Wanner et al., 2012), severely limit the potential for clinical translatability of this research. The pharmacological modulation of TRPV1 activity as a therapeutic strategy is further complicated by its expression in sensory neurons, where channel activation is associated with significant pain. Consequently, while TRPV1 appears to play a role in sepsis pathogenesis, it may not represent a viable clinical target.

CHAPTER 5 | IN VITRO CHARACTERISATION OF VASCULAR TRPV1 AND TRPV4

5.1 Introduction

Numerous published reports (Clark et al., 2007, Wang et al., 2008a) and data presented in **Chapter 4** suggest that TRPV1 may confer cardiovascular protection in sepsis. While the neurogenic effects of TRPV1 activation on vascular function are well established, however, the contribution of vascular TRPV1 expression to these responses is unknown. There has been much recent evidence suggesting that TRPV1 may also contribute to vascular function through non-neuronal mechanisms, though studies reporting its expression in endothelial and smooth muscle tissue have often raised contention.

5.1.1 Functional expression of TRPV1 in the endothelium

Functional expression of TRPV1 in the endothelium was initially inferred from recordings of capsaicin-induced Ca^{2+} influx in primary cultures of human cerebral artery endothelial cells (ECs) (Golech et al., 2004), although biochemical analysis of TRPV1 mRNA levels has produced conflicting results. TRPV1 has been detected in human pulmonary artery ECs (Fantozzi et al., 2003) and murine aortic ECs (Yang et al., 2010b), as well as in the endothelium of intact murine mesenteric arteries (Yang et al., 2010b) and porcine coronary arteries (Bratz et al., 2008), though other investigators failed to detect TRPV1 mRNA in the endothelium of rat middle cerebral arteries (Marrelli et al., 2007). Whether discrepancies are due to changes in experimental conditions, or species or tissue differences, is unclear.

The vascular endothelium produces a number of vasoactive substances, of which the main vasodilatory mediators are nitric oxide (NO), prostacyclin (PGI_2) and endothelium-derived hyperpolarising factor (EDHF). The identity of the latter is debated and may vary between vascular beds, though K^+ efflux through endothelial intermediate K^+ channels and large conductance K^+ channels is thought to be an important contributor. Activation of these vasodilatory mediators is highly dependent on Ca^{2+} , and it is thought that influx of Ca^{2+} through endothelial TRPV1 is sufficient to stimulate each of these pathways (Baylie and Brayden, 2011). Correspondingly, activation of TRPV1 in the vascular endothelium has been reported to cause vasorelaxation in a number of studies (Baylie and Brayden, 2011, Bratz et al., 2008, Hoi et al., 2007, Kark et al., 2008, Yang et al., 2010a).

Kark and colleagues studied this effect using a preparation of rat skeletal muscle arteries, in which perivascular innervation is absent, allowing delineation of functional vascular TRPV1 expression without contamination from neuronal input (Kark et al., 2008). Administration of low-dose capsaicin (10 nM) produced an endothelium-dependent vasorelaxation that was abolished by the endothelial nitric oxide synthase (eNOS) inhibitor N^ω-nitro-L-arginine methyl ester (L-NAME) as well as by removal of the endothelium itself, suggesting that activation of endothelial TRPV1 is capable of causing NO-dependent vasodilatation (Kark et al., 2008). Conversely, at higher concentrations (0.1–1 μM), capsaicin caused endothelium-independent vasoconstriction, that was attributed to functional expression of TRPV1 in vascular smooth muscle cells (VSMCs) (Kark et al., 2008).

TRPV1-mediated endothelium-dependent vasodilatation was also demonstrated in porcine coronary arteries, where capsaicin caused a dose-dependent vasorelaxation, and at 100 μM induced cation influx in freshly isolated ECs (Bratz *et al.*, 2008). This effect was abolished in endothelium-denuded rings and in the presence of the selective TRPV1 antagonist capsazepine. Furthermore, L-NAME, iberiotoxin (a blocker of large conductance K⁺ channels) and tetraethylammonium (TEA; a general K⁺ channel blocker) significantly reduced the vasodilatory response to capsaicin, though the effect was not completely abolished. Whether this was due to non-specific effects of capsaicin, or to endothelium-independent mechanisms was not investigated in this study, though it is clear that both NO and K⁺ channel activation contribute to capsaicin-induced vasorelaxation (Bratz *et al.*, 2008).

It should be noted, however, that at concentrations exceeding 10 μM, capsaicin can produce TRPV1-independent effects, and can alter the function of a number of structurally unrelated proteins; furthermore, many of these are also modulated by the antagonist capsazepine (Lundbaek et al., 2005). Moreover, high concentrations of capsaicin have been shown to cause membrane perturbations (Aranda et al., 1995, Meddings et al., 1991), which could induce Ca²⁺ influx independently of TRPV1.

Other studies also support a role for TRPV1-mediated endothelial activation, however. VSN16, a synthetic cannabinoid-like compound, was shown to cause endothelium-dependent capsazepine-sensitive relaxation of rat mesenteric arteries (Hoi et al., 2007). Furthermore, in a chronically denervated mesenteric vascular bed, perfusion of nanomolar concentrations of capsaicin and anandamide led to an increase in perfusate NO levels, which was abolished by capsazepine and L-NAME (Poblete et al., 2005). The selective TRPV1 antagonist SB366791 also inhibited endothelium-dependent NO release by capsaicin and anandamide in non-denervated

arteries, indicating a specific role for TRPV1 in this response. Although anandamide has also been shown to activate TRPV4, (Watanabe et al., 2003b) this pathway was ruled out as a mechanism for anandamide-induced vasodilatation in this bed, following the observation that an alternative TRPV4 agonist, 4 α -PDD, failed to elicit significant NO production (Poblete et al., 2005).

A more recent investigation by Yang and colleagues, using a combination of *in vitro* and *in vivo* techniques, has provided further evidence of functional TRPV1 expression in the vascular endothelium. The authors demonstrated a capsaicin-induced Ca²⁺ influx in primary cultures of mouse aortic ECs, which increased phosphorylation and activation of protein kinase A (PKA) and eNOS (Yang et al., 2010b). In addition to causing vasodilatation directly, through phosphorylation and inhibition of myosin light chain kinase (Conti and Adelstein, 1980), PKA also promotes increased NO production through phosphorylation of eNOS, enhancing smooth muscle relaxation (Michell et al., 2001). Capsaicin-induced endothelium-dependent NO production and vasodilatation in isolated mouse mesenteric arteries were significantly attenuated in TRPV1 knockout (KO) animals. Furthermore, this study demonstrated for the first time that chronic dietary administration of capsaicin can attenuate the haemodynamic phenotype of spontaneously hypertensive rats (SHRs), and improve their endothelial function, evidenced by increased vasodilatory responses to acetylcholine (ACh) (Yang et al., 2010b). Improved ACh-mediated vasorelaxation was not observed in capsaicin-fed TRPV1 KO mice and was similarly inhibited by L-NAME. Moreover, increased levels of phosphorylated PKA, phosphorylated eNOS and NO, observed in wildtype (WT) mice, were not found in KO counterparts.

Given that TRPV1 shows broad tissue distribution, it is unclear whether endothelial TRPV1 is truly responsible for the anti-hypertensive effects of capsaicin *in vivo*. Furthermore, given that chronic exposure of the channel to capsaicin can both desensitise and downregulate the channel, and defunctionalise sensory nerve terminals, it is not clear whether dietary administration of capsaicin would induce chronic channel activation or inhibition of channel function. However, the *in vitro* data published by Yang and colleagues, from isolated ECs and mesenteric arteries provide strong evidence that TRPV1 is present and functional within the endothelium, and that activation leads to Ca²⁺ influx, PKA activation, NO production and smooth muscle relaxation (Yang et al., 2010b). These results, coupled with the separate observation that neonatal sensory denervation does not inhibit capsaicin-induced NO release

(Rocha and Bendhack, 2009), are strongly indicative of a non-neuronal mechanism of TRPV1-mediated vasodilatation.

In addition to stimulating increased NO production, TRPV1 may also elicit vasorelaxation through alternative pathways. A TRPV1-mediated increase in endothelium-derived CGRP expression and secretion has been shown to occur in human umbilical vein ECs (HUVECs) (Luo et al., 2008), as well as in rat mesenteric and aortic ECs (Ye et al., 2007), though whether this pathway represents a physiological mechanism for arterial tone regulation is not clear. Alternatively, activation of TRPV1 in human cerebral artery ECs by 2-arachidonoyl-glycerol (2-AG) has been shown to cause vasorelaxation through the phosphorylation of vasodilator-stimulated phosphoprotein (VASP) (Golech *et al.*, 2004). It is possible that activation of endothelial TRPV1 stimulates a Ca^{2+} -dependent phosphorylation of PKA and/or PKG [consistent with previous observations (Yang et al., 2010b)], which mediate vasodilatation through phosphorylation of VASP, a known substrate of PKA and PKG (Golech *et al.*, 2004).

5.1.2 Functional expression of TRPV1 in vascular smooth muscle

While activation of endothelial TRPV1 has been associated with vascular relaxation, there have been several reports of capsaicin-induced vasoconstriction that may represent functional expression of TRPV1 in VSMCs. Investigations of this hypothesis, however, have produced somewhat conflicting results. Yang and colleagues have demonstrated the presence of TRPV1 mRNA in endothelium-denuded rat aorta and intralobal arteries (Yang et al., 2010b), which the authors interpreted as an indication of VSMC expression, though contamination by perivascular nerves or residual ECs was not ruled out. Furthermore, although other investigators have inferred protein expression of TRPV1 in vascular smooth muscle from functional assays, these studies have been limited by a lack of biochemical data (Kark et al., 2008, Bratz et al., 2008) or by the use of cultured and immortalised cell lines (Wang et al., 2008b). One group failed to detect any TRPV1 expression in rat cerebral artery smooth muscle (Marrelli et al., 2007).

To date, four groups have published robust evidence of functional TRPV1 expression in VSMCs. Using an isolated rat hind limb preparation, Kark and colleagues demonstrated increased vascular resistance and decreased skeletal muscle perfusion in response to capsaicin, that was unaffected by sensory denervation (Kark *et al.*, 2008). Furthermore, in isolated rat skeletal muscle arterioles, which are devoid of sensory innervation, higher doses of capsaicin (0.1–1 μM) elicited endothelium-independent vasoconstriction (Kark *et al.*, 2008). This vasoconstriction was later found to be absent in arterioles treated with the selective TRPV1

antagonist AMG-9810 or isolated from TRPV1 KO mice, and was additionally shown to occur in parallel with Ca^{2+} influx into the arteriolar wall through simultaneous measurement of vessel diameter and intracellular Ca^{2+} levels (Czikora *et al.*, 2012). Correspondingly, expression of TRPV1 in rat aortic smooth muscle was detected both by qRT-PCR and immunohistochemical analysis (Kark *et al.*, 2008), and was inferred from observations of significant capsaicin-induced Ca^{2+} influx in isolated canine coronary artery SMCs (Czikora *et al.*, 2012).

Cavanaugh *et al.* used gene targeting to modify the TRPV1 genetic locus, generating reporter mice that produce a highly sensitive read-out of TRPV1 expression (Cavanaugh *et al.*, 2011). In contrast to the observations of Kark and colleagues, this group failed to detect any TRPV1 protein in the murine aorta, though TRPV1 was detected in VSMCs of a number of thermoregulatory tissues, including the cremaster muscle, the dura, the tongue, the trachea, the ear and the skin (Cavanaugh *et al.*, 2011). In these tissues, VSMC TRPV1 expression was restricted to small- to medium-diameter vessels, postulated to represent small resistance arteries and arterioles, and was substantially reduced after RTX-induced degradation of TRPV1-positive cells. Confirmation of functional TRPV1 expression was achieved by live-cell calcium imaging of isolated and denervated ear arterioles. Importantly, capsaicin failed to produce either Ca^{2+} influx or vasoconstriction in isolated arterioles from TRPV1 KO mice (Cavanaugh *et al.*, 2011).

In 2011, Ma and colleagues reported TRPV1 mRNA and protein expression in murine aorta and freshly isolated aortic VSMCs by a variety of biochemical techniques, confirming functional expression by Fura-2-based calcium imaging (Ma *et al.*, 2011). More recently, Martin *et al.* demonstrated TRPV1 mRNA and protein expression in rat intrapulmonary arteries, as well as Ca^{2+} influx in isolated pulmonary artery SMC, in response to 10 μM capsaicin (Martin *et al.*, 2012). In this study, both TRPV1 and TRPV4 were suggested to contribute directly to SMC migration (Martin *et al.*, 2012).

Despite discrepancies between different studies, that may represent species differences or insufficiently selective antibodies or agonist concentrations, collectively these reports provide compelling evidence that TRPV1 is present and functional in VSMCs, though perhaps in a regionally restricted fashion. It is possible that activation of TRPV1 on VSMCs could counterbalance the effects of neuronal TRPV1 activation by physiological agonists, such as heat and protons, which supports the observations of capsaicin-induced vasoconstriction, reported previously (Scotland *et al.*, 2004).

5.1.3 A role for TRPV4 in vascular regulation

While this study was originally designed to investigate the role of TRPV1 in vascular regulation, we were also aware of an increasing research interest in other members of the TRP vanilloid subfamily, most notably TRPV4. TRPV4 expression in both endothelial and smooth muscle tissues is well established (Baylie and Brayden, 2011), and recent reports linking excessive activation of TRPV4 to profound hypotension, endothelial failure and circulatory collapse (Willette et al., 2008, Sonkusare et al., 2012) suggest that it may play a role in the haemodynamic dysfunction that characterises septic shock. In light of this evidence, we have additionally characterised endothelial TRPV4 activity in basal and inflammatory conditions *in vitro*, with a view to extending this research *in vivo*, as described in **Chapter 6**. The molecular basis of TRPV4-mediated vasoactivity is also described more extensively in **Chapter 6**.

5.1.4 Hypotheses

Based on previous reports, as well as our own data supporting a vasculo-protective role for TRPV1 in sepsis, it was hypothesised that TRPV1 may mediate this protection through activity in the vascular system. Since peripheral vasodilatation may confer protection against perfusion impairment in sepsis, we hypothesised that TRPV1 may be expressed in vascular tissue, and that alterations in its expression and/or activity in sepsis may contribute to the maintenance of perfusion in regional beds.

In light of recent reports linking excessive TRPV4 activation to profound hypotension and circulatory collapse (key features of sepsis) we additionally hypothesised that vascular TRPV4 activity may be altered under inflammatory conditions.

5.1.5 Aims

Based on the hypotheses outlined above, and the limitations of previous studies of vascular TRPV1 expression, our aims were as follows:

- 1) To address the controversy regarding vascular TRPV1 expression in published literature, and to determine whether TRPV1 is expressed in vascular tissues and cells from different species
- 2) To confirm the expression of TRPV4 in vascular cells and tissues
- 3) To characterise TRPV1 and TRPV4 activity in isolated vascular cell types under basal and inflammatory conditions using fluorometric calcium imaging techniques
- 4) To characterise TRPV1 and TRPV4 activity in macrophages, based on previous reports of TRPV1- and TRPV4-induced macrophage-mediated inflammatory function

5.2 Methods

5.2.1 Materials

A list of materials used in this study, and details of their origin, are provided in **Table 9**.

Material	Manufacturer	Catalogue No.
Capsaicin	Sigma-Aldrich	M2028
GSK1016790A	Sigma-Aldrich	G0798
HC-067047	Tocris	4100
Lipopolysaccharide from <i>Salmonella typhimurium</i>	Sigma-Aldrich	L7261
DMEM (4.5 g/L glucose)	PAA Laboratories	E15-009
DMEM (1 g/L glucose)	PAA Laboratories	E15-005
Endothelial cell medium	PAA Laboratories	U15-002
HBSS (with Ca ²⁺ and Mg ²⁺)	Sigma-Aldrich	55037C
Ionomycin	Sigma-Aldrich	I9657
Adenosine triphosphate	Sigma-Aldrich	A2383
Thiozoly blue tetrazolium bromide	Sigma-Aldrich	M5655

Table 9. Materials used in study. DMEM: Dulbecco's modified Eagle's medium, HBSS: Hank's balanced salt solution

5.2.2 Cell isolation and culture

Primary bovine aortic endothelial cells, murine pulmonary endothelial cells and murine aortic smooth muscle cells, immortalised murine skin endothelioma cells and murine bone marrow-derived macrophages were cultured as described in section **2.8**.

5.2.3 Measurement of vascular TRPV1 and TRPV4 expression

Non-quantitative assessment of TRPV1 and TRPV4 mRNA expression in vascular cells and isolated murine aortae was performed as described in section **2.9**. Vascular TRPV1 expression was also evaluated by Western immunoblotting, as described in section **2.7**. A list of antibodies used is provided in **Table 10**.

Antibody	Manufacturer	Catalogue No	Species	Vehicle	Working dilution
<i>Primary antibodies</i>					
Anti-TRPV1	Alomone Labs	ACC-030	Rabbit	1% BSA, 0.05% NaN ₃ in PBS	1 : 200
Anti-TRPV1	Abcam	Ab45759	Mouse	1% BSA, 0.05% NaN ₃ in PBS	1 : 200
Anti-TRPV1	Donated by Prof M. Caterina, Johns Hopkins, USA		Rabbit	1% BSA, 0.05% NaN ₃ in PBS	1 : 500
Anti-TRPV1	Sigma	V2764	Rabbit	1% BSA, 0.05% NaN ₃ in PBS	1 : 500
Anti-β-actin	Sigma	A2228	Mouse	1% non-fat milk in TBS-T	1 : 2000
<i>Secondary antibodies</i>					
Anti-rabbit	Cell Signaling	7074		1% non-fat milk in TBS-T	1 : 2000
Anti-mouse	Sigma	A4416		1% non-fat milk in TBS-T	1 : 5000

Table 10. Antibodies used in Western blot analysis. Ab45759 (Abcam), A2228 (Sigma) and the anti-TRPV1 antibody donated by Prof Caterina were monoclonal; all others were polyclonal. TBS-T: Tris-buffered saline containing 0.1% Tween-20.

5.2.4 Measurement of vascular TRPV1 and TRPV4 activity

5.2.4.1 Calcium fluorometry

As both TRPV1 and TRPV4 are cation channels highly permeable to Ca^{2+} , a fluorescent Ca^{2+} indicator was used to investigate the functional expression of these two receptors in endothelial cells. In the wake of pioneering work by Nobel laureate Professor Roger Tsien in the 1980s, the development of highly accurate fluorescent calcium indicator dyes has progressed steadily. These dyes are generally based on a Ca^{2+} -sensitive construct coupled to fluorophore, which upon binding to Ca^{2+} ions undergoes a conformational change, eliciting a fluorescent signal.

More recently, Ca^{2+} dyes have been engineered as acetoxymethyl (AM) esters, which allow loading of relatively hydrophilic dyes across hydrophobic cellular membranes by passive diffusion, negating the need for microinjection or electroporation previously required (Paredes et al., 2008). Following diffusion across the plasma membrane, AM groups are cleaved by intracellular esterases, effectively trapping the indicator inside the cell.

Vascular TRPV1 and TRPV4 activity was assessed by Fura-2/AM either in a microscope-based single cell imaging set-up, or by a microplate-based method, as described in section 2.11. Fura-2/AM is one of the ratiometric indicators, which shift the peak wavelength of their excitation or emission curve upon binding Ca^{2+} ions. Specifically, Fura-2 is a dual-excitation dye: peak absorbance shifts from 380 nm in the Ca^{2+} -free state, to 340 nm following Ca^{2+} binding (**Fig. 33**). Peak emission occurs at 510 nm for both excitation wavelengths (Paredes et al., 2008). A significant advantage of using ratiometric dyes is that they correct for artefacts associated with photobleaching, changes in culture volume, uneven dye loading, leakage or extrusion.

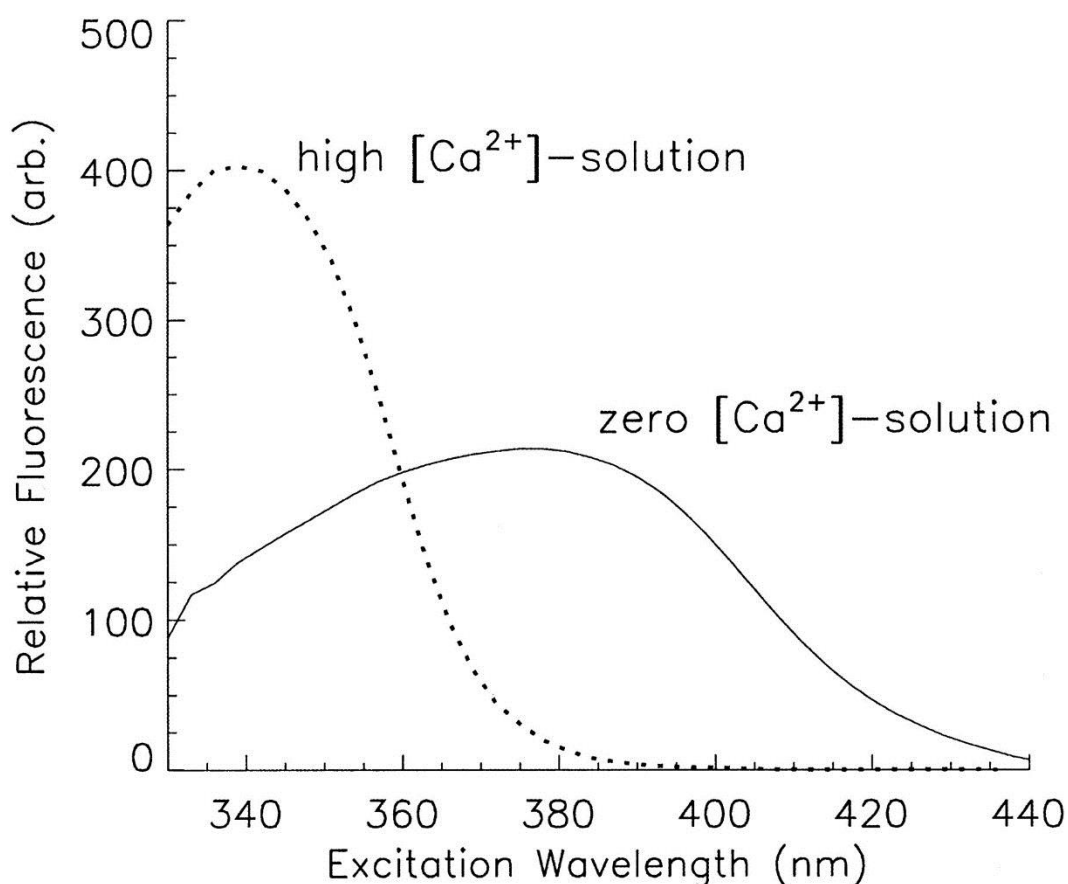


Figure 33. Excitation spectra for Fura-2 fluorescence recorded along the 510 nm emission wavelength in high [Ca²⁺] and zero [Ca²⁺] solutions. Peak fluorescence was recorded at 376 nm and 339 nm, respectively. Figure adapted from Palmer and Moore (Palmer and Moore, 2000).

5.2.4.2 Experimental set-up

All Ca²⁺-fluorometry was conducted using either a high-throughput microplate-based imaging system, or a microscope-based technique adapted for live imaging of single cells, as described in section 2.11. Briefly, cells were plated either into 96-well plates or onto glass coverslips, and allowed to reach approximately 80% confluence. Culture medium was then replaced with Hank's balanced salt solution (HBSS; with 10 mM glucose, 10 mM HEPES, pH 7.4) containing 2.5 μM Fura-2/AM and 2 mM probenecid, and cells were incubated at 37°C for 1 h. Fluorescence was then recorded over a baseline, and following the addition of agonists.

5.2.4.3 Assay validation

We aimed to probe TRPV1 and TRPV4 channel activity in endothelial cells using the selective agonists, capsaicin and GSK1016790A, respectively. While the latter is consistently administered in a vehicle of 1-2% DMSO (Alexander et al., 2013, Willette et al., 2008, Pankey et al., 2014), capsaicin preparations were found to vary between previous studies. In order to

determine the optimal preparation for *in vitro* capsaicin delivery in this study, a range of capsaicin concentrations was dissolved in three different commonly used vehicles, and applied to HEK cells stably expressing TRPV1 (HEK-TRPV1; kindly donated by Dr Andy Grant). Fluorescence was read on a microplate imaging system, as described in section 2.11.1, and capsaicin was added, after 20-s baseline recording, by an automated pipettor system. Capsaicin in both 2% DMSO and 2% ethanol/2% Tween-80 caused a concentration-dependent increase in relative fluorescence (**Fig. 34a and 34c, respectively**). Capsaicin in 2% ethanol, on the other hand, did not appear to induce concentration-dependent calcium influx in HEK-TRPV1 cells, suggesting insufficient drug solubility (**Fig. 34b**). Unfortunately, maximum response and EC₅₀ values for the different treatments were not calculated, as some curves were not considered to have reached maximum response.

We additionally sought to assess drug selectivity at higher concentrations using untransfected HEK cells and an identical microplate-based system. Neither capsaicin in 2% DMSO nor capsaicin in 2% ethanol produced any response in untransfected cells at concentrations of 10 µM and below (**Fig. 35a and 35b, respectively**). Similarly, the TRPV4 agonist GSK1016790A produced no response in HEK cells at 10 µM and below (**Fig. 35d**). Capsaicin dissolved in 2% ethanol/2% Tween-80, on the other hand, produced a small and relatively consistent calcium influx at all concentrations, suggesting a non-specific vehicle-mediated effect (**Fig. 35c**). In light of these results, 2% DMSO was considered the optimal vehicle for *in vitro* capsaicin administration, and was used in all further experiments.

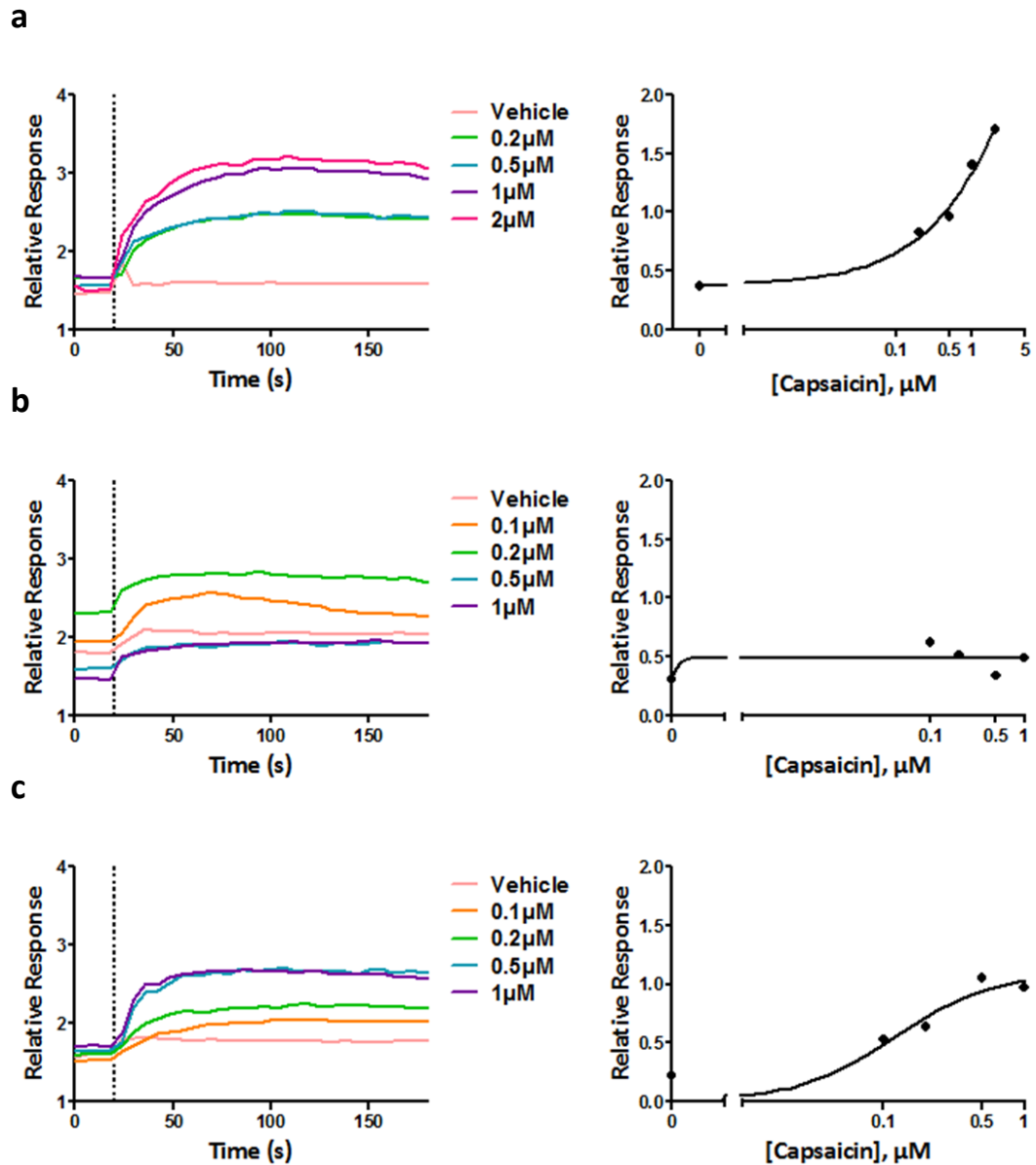


Figure 34. Assessment of capsaicin solubility in three different vehicles. Concentration-response curves were generated in HEK cells stably transfected with rat TRPV1, treated with increasing doses of capsaicin (0.1 – 2 μM) dissolved in 3 different vehicles: **(a)** 2% DMSO, **(b)** 2% ethanol or **(c)** 2% ethanol + 2% Tween-80. Left hand panel: representative traces of ratiometric emission intensity over time. Drugs were added after 20 s, denoted by the dotted line. Right hand panel: corresponding non-linear regression curves generated for each treatment. Data are presented as mean response over 3 wells for each concentration.

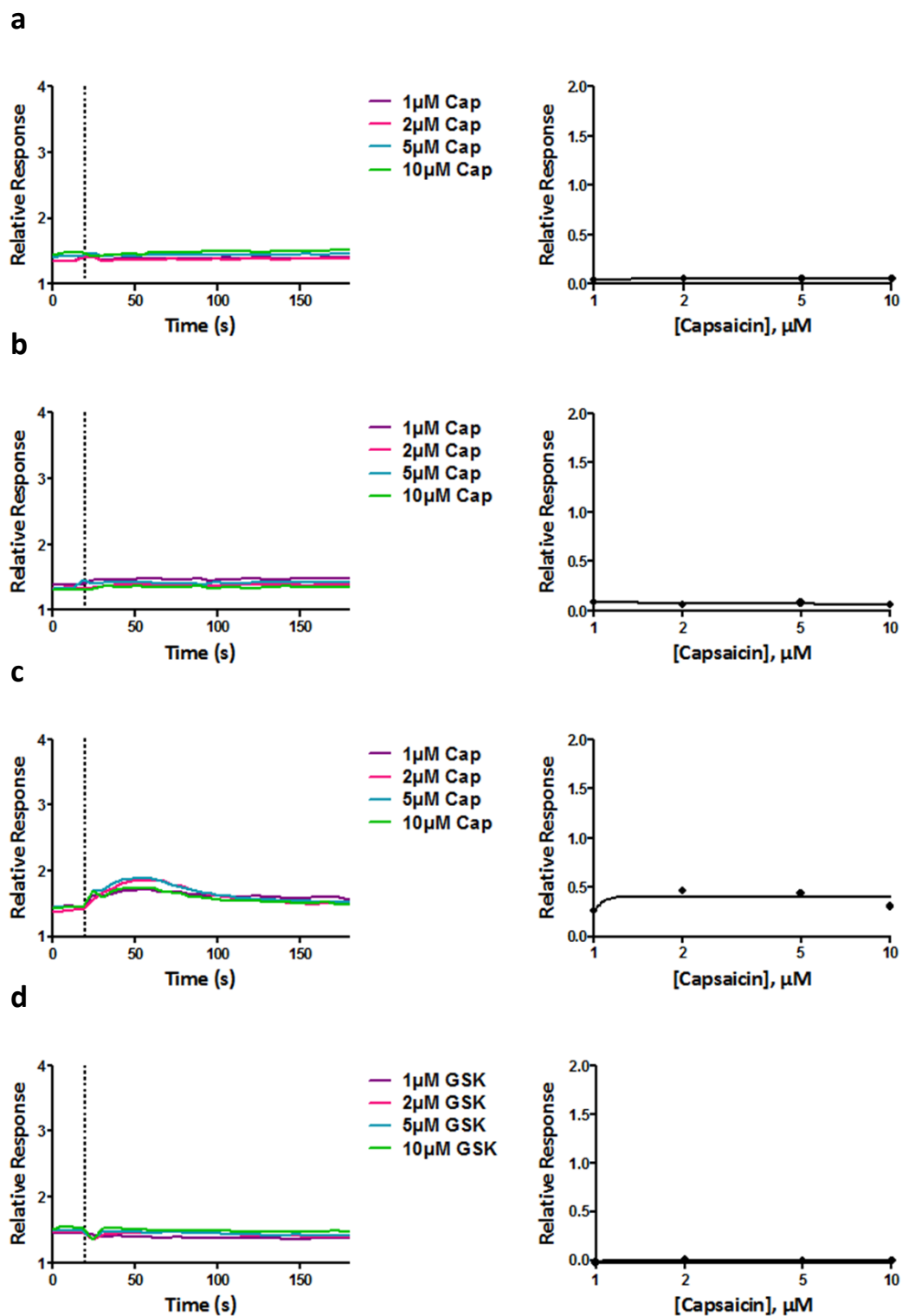


Figure 35. Assessment of drug selectivity in untransfected HEK cells. Concentration-response curves were generated in untransfected HEK cells treated with increasing doses of capsaicin (1–10 μ M; Cap) dissolved in 3 different vehicles: **(a)** 2% DMSO; **(b)** 2% ethanol; or **(c)** 2% ethanol + 2% Tween-80, or with GSK1016790A (1–10 μ M; GSK) in 2% DMSO **(d)**. Left hand panel: representative traces of ratiometric emission intensity over time. Drugs were added after 20 s, as denoted by the dotted line. Right hand panel: corresponding non-linear regression curves. Data are presented as mean response over 3 wells for each concentration.

5.2.5 Sequencing of TRPV1 KO cDNA

RNA was extracted from lungs of TRPV1 KO mice and reverse-transcribed into cDNA, as described in section 2.9.1. PCR amplification of cDNA was performed using the TRPV1 KO forward primer and TRPV1 common reverse primer, as described in section 2.1.3. PCR products were separated on a 1% agarose gel by electrophoresis (80 V, 50 min). Bands were excised using a sterile scalpel, and DNA was extracted using QIAquick gel extraction kit (Qiagen, 28704), as per manufacturer's instructions. DNA was eluted in elution buffer (10 mM Tris-Cl, pH 8.5), and sequenced using the following primers:

- a. Neomycin forward: 5'-TGG ATG TGG AAT GTG TGC GAG-3
- b. TRPV1 C-terminus reverse: 5'-TCC TCA TGC ACT TCA GGA AA-3'

Sequencing was performed at the Wolfson Institute for Biomedical Research, University College London.

5.2.6 Assessment of cell viability by MTT assay

Viability of bovine aortic endothelial cells (bAEC) following exposure to increasing concentrations of LPS over 24 h was assessed by MTT assay. This colorimetric assay was developed in 1983 and is based on the tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], which is reduced to dark blue formazan by the mitochondria of living cells, but not of dead cells or erythrocytes (Mosmann, 1983). Dissolution of this coloured crystalline product produces a homogenous solution that is suitable for spectrophotometric analysis.

bAEC were plated in 96-well plates and allowed to reach 80% confluency. They were then treated with increasing concentrations of LPS (1–100 ng/mL) in the presence of either TRPV4 antagonist HC-067047 (10 μ M) or vehicle (2% DMSO in DMEM). After 24 h, 10 μ L MTT (in saline) was then added to each well (final concentration: 5 mg/mL), and cells were incubated at 37°C for 4 h. The formazan product was solubilised in DMSO, and absorbance was read at 560 nm in a FlexStation® 3 Microplate Reader (Molecular Devices, Sunnydale, CA).

5.2.7 Measurement of macrophage TRPV1 and TRPV4 activity

Bone marrow-derived macrophages (BMDM) were isolated from the femur bone of TRPV4 WT and KO mice, and were plated at a density of 6×10^5 /well in eight-chamber culture slides (BD Falcon, 354118). 1- μ m fluorescent latex beads (1:1000; 5 μ l/well; Sigma-Aldrich, L2778) were added to cells in the presence or absence of LPS (100 ng/ml) with either GSK1016790A (100 nM) or vehicle (2% DMSO). After 1 h incubation at 37°C, extracellular beads were removed by washing (3 x phosphate buffered saline; PBS), and cells were fixed in 4% paraformaldehyde for 10 min at RT. Wells were then washed again (3 x PBS), the chamber walls were removed, and 10 μ L PBS was pipetted into the centre of each chamber. A glass coverslip was placed over the slide and sealed with varnish. A fluorescent microscope was then used to capture an image from the centre of each well, and fluorescence was quantified using ImageJ software (National Institutes of Health). Results are expressed as fluorescence as percentage of total area.

5.3 Results

5.3.1 TRPV1 and TRPV4 mRNA is expressed in vascular endothelium

Total RNA was isolated from murine aortae and endothelial cells from three different species and was reverse-transcribed to cDNA. TRPV1 and TRPV4 cDNA was then amplified by PCR thermocycling with specific primers. TRPV1 and TRPV4 mRNA was detected in murine aortic tissue (**Fig. 36a**) and all three endothelial cell types: immortalised murine skin endothelioma cells (sEnd1), primary human umbilical vein endothelial cells (HUVEC) and bovine aortic endothelial cells (bAEC; **Fig. 36b**). In bAEC, expression of both genes was still preserved at passage 7, consistent with a previous report demonstrating a persistent endothelial phenotype for up to 35 population doublings (Augustin-Voss et al., 1993).

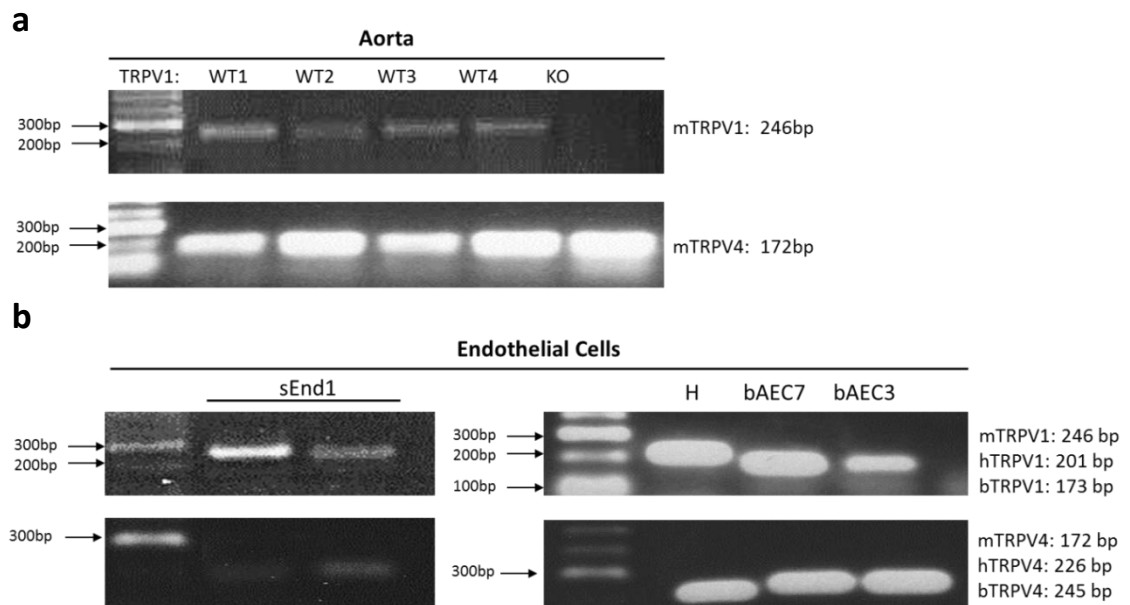


Figure 36. Agarose gels visualised under UV illumination showing products of PCR amplification of TRPV1 and TRPV4 cDNA. (a) Expression of murine TRPV1 (mTRPV1) and TRPV4 (mTRPV4) mRNA in aortic lysates from 4 TRPV1 WT mice and one TRPV1 KO mouse. **(b)** Expression of murine, human (h) and bovine (b) TRPV1 and TRPV4 in mouse skin endothelioma cells (sEnd1), human umbilical vein endothelial cells (H) and bovine aortic endothelial cells at passage 7 (bAEC7) and passage 3 (bAEC 3).

5.3.2 TRPV1 protein expression is not detectable with commercially available antibodies

Whereas functional endothelial expression of TRPV4 is now well established (Sonkusare *et al.*, 2012), the role and indeed the presence of TRPV1 in these cells remains contentious. Although numerous reports of endothelial TRPV1 expression have been published to date, studies have typically relied on inferences based on mRNA data or on unvalidated antibodies, often used in the absence of appropriate positive and negative controls. Despite observing clear TRPV1 mRNA expression in aortic and endothelial lysates, we were unable to demonstrate TRPV1 protein expression by traditional antibody-recognition methods. Protein samples from TRPV1 WT and KO mice were size-separated by SDS-PAGE, and transferred to PVDF membranes for incubation with an anti-TRPV1 antibody (Alomone, ACC-030), previously suggested to bind selectively to TRPV1 WT protein (Yang *et al.*, 2010b). In this instance, however, a single band of approximately 80 kDa was observed in aortic and dorsal root ganglia (DRG) samples from both WT and KO mice (**Fig. 37**). This was repeated using samples from further WT and KO mice (n = 12) and in each case an identical positive band was observed in both WT and KO samples, indicative of non-specific binding.

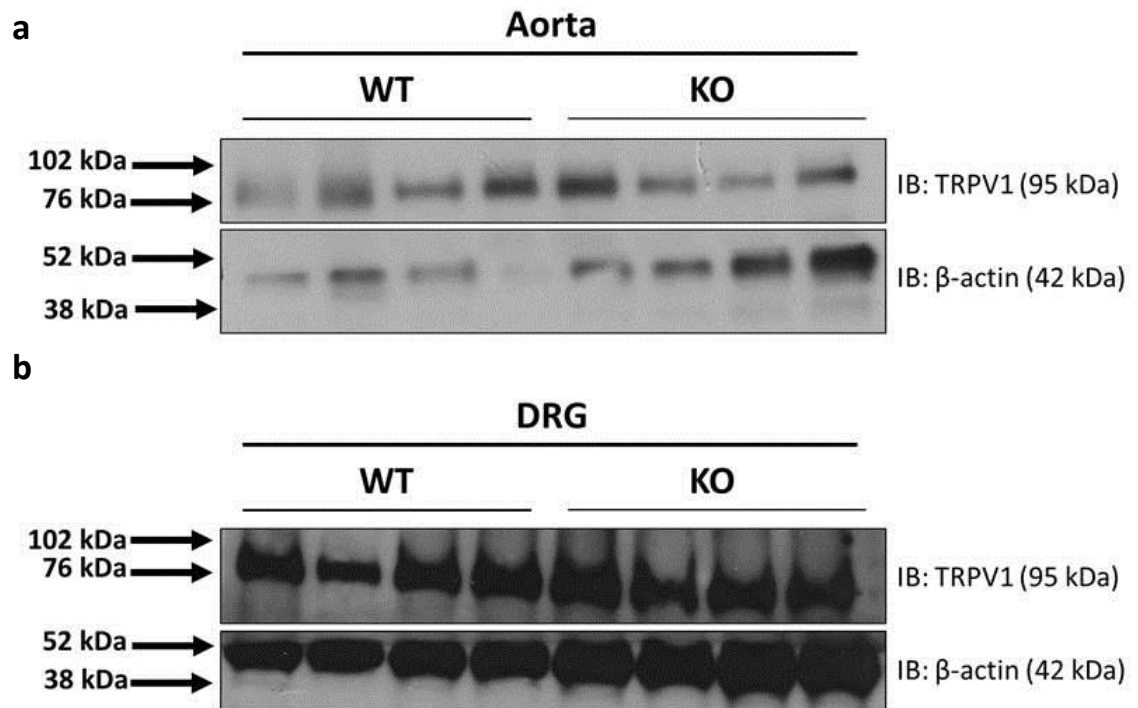


Figure 37. Representative immunoblots of TRPV1 protein expression in murine tissues. (a) and (b) Immunopositive bands in aortic and DRG lysates, respectively, from TRPV1 WT and KO mice, probed with ACC-030 anti-TRPV1 antibody (Alomone; predicted molecular weight 95 kDa). β -actin expression was used as loading control (predicted molecular weight 42 kDa). The presence of immunoreactive bands in TRPV1 KO samples indicates that these bands are likely false positives, and suggests that this antibody is reactive with a non-specific protein slightly smaller than TRPV1 within the tissue lysate.

5.3.2.1 Sequencing of TRPV1 KO cDNA

The ACC-030 anti-TRPV1 antibody used for immunoblotting was raised against the extreme C-terminus of TRPV1. Given that generation of the mutant TRPV1 construct involves replacement of transmembrane domains 5 and 6 with a neomycin cassette, rather than deletion of the entire C-terminus (Caterina et al., 2000), cDNA isolated from the lungs of TRPV1 KO mice was sequenced, in order to determine whether the C-terminus of the mutant protein is still translated. TRPV1 KO lung cDNA was isolated, and a region spanning the neomycin cassette and the C-terminus of TRPV1 was amplified by PCR and sequenced. The C-terminal end of the neomycin cassette was found to contain a TAG stop codon, precluding the possibility of C-terminal translation in mutant TRPV1 (**Fig. 38**), and invalidating the use of the ACC-030 antibody for specific identification of TRPV1.

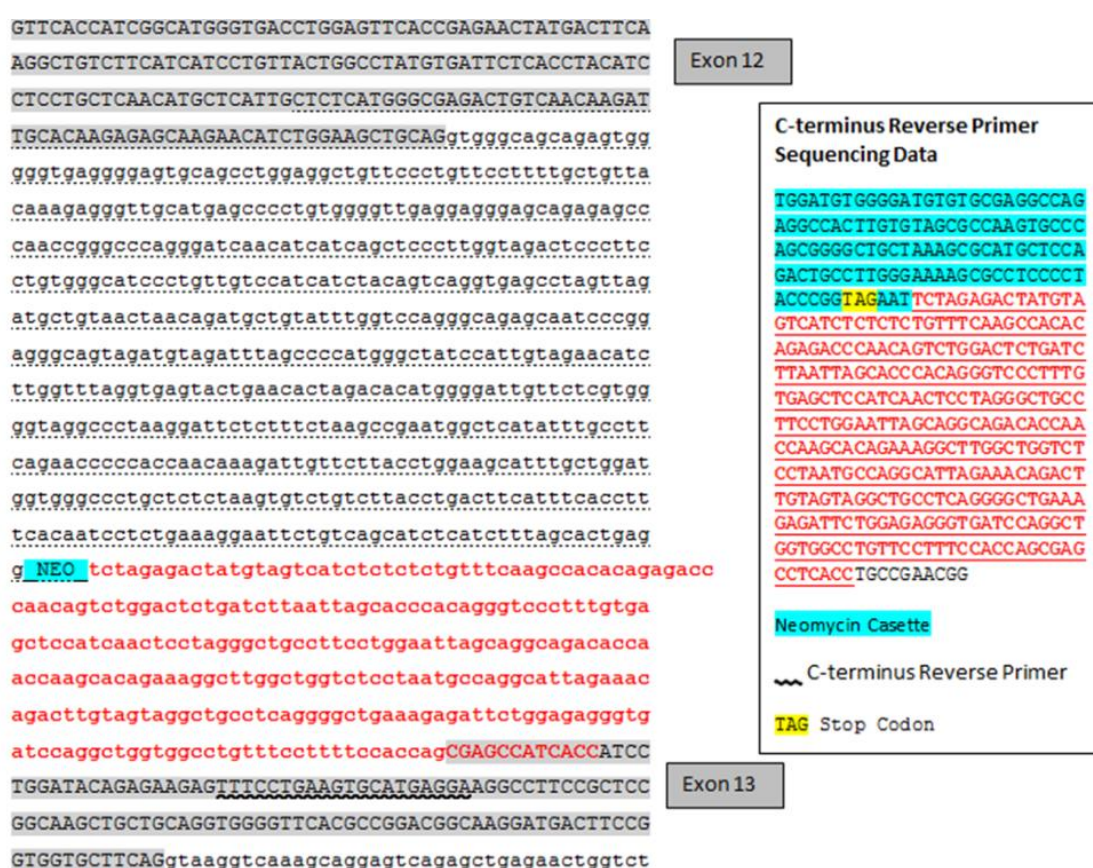


Figure 38. Sequencing data from mutant TRPV1 lung cDNA. The sequence obtained from PCR amplification of mutant cDNA using a forward primer binding in the inserted neomycin cassette and a reverse primer binding in the C-terminus of TRPV1 is shown in the box. Nucleotides highlighted in blue represent the neomycin cassette; those in red correspond to WT TRPV1 sequence. Alignment of this sequence with that of WT TRPV1, shown on the left, demonstrates transcription of WT DNA downstream of the inserted neomycin cassette (NEO). Overlapping sequence is highlighted in red, and the binding site of the C-terminal reverse primer in exon 13 is underlined. The TAG stop codon (highlighted in yellow) in the C-terminus of the neomycin cassette suggests that TRPV1 C-terminus mRNA is not translated.

5.3.2.2 Characterisation of other TRPV1 antibodies

In addition to ACC-030 (Alomone Labs), a number of other antibodies were tested, each also producing an immunoreactive band in TRPV1 KO tissue, and often non-specific bands of incorrect molecular weight. Representative immunoblots of TRPV1 WT and KO tissue using various commercially available and donated anti-TRPV1 antibodies are shown in **Fig. 39**. A second batch of the original ACC-030 antibody was also tested. The details of all antibodies used are provided in **Table 10**.

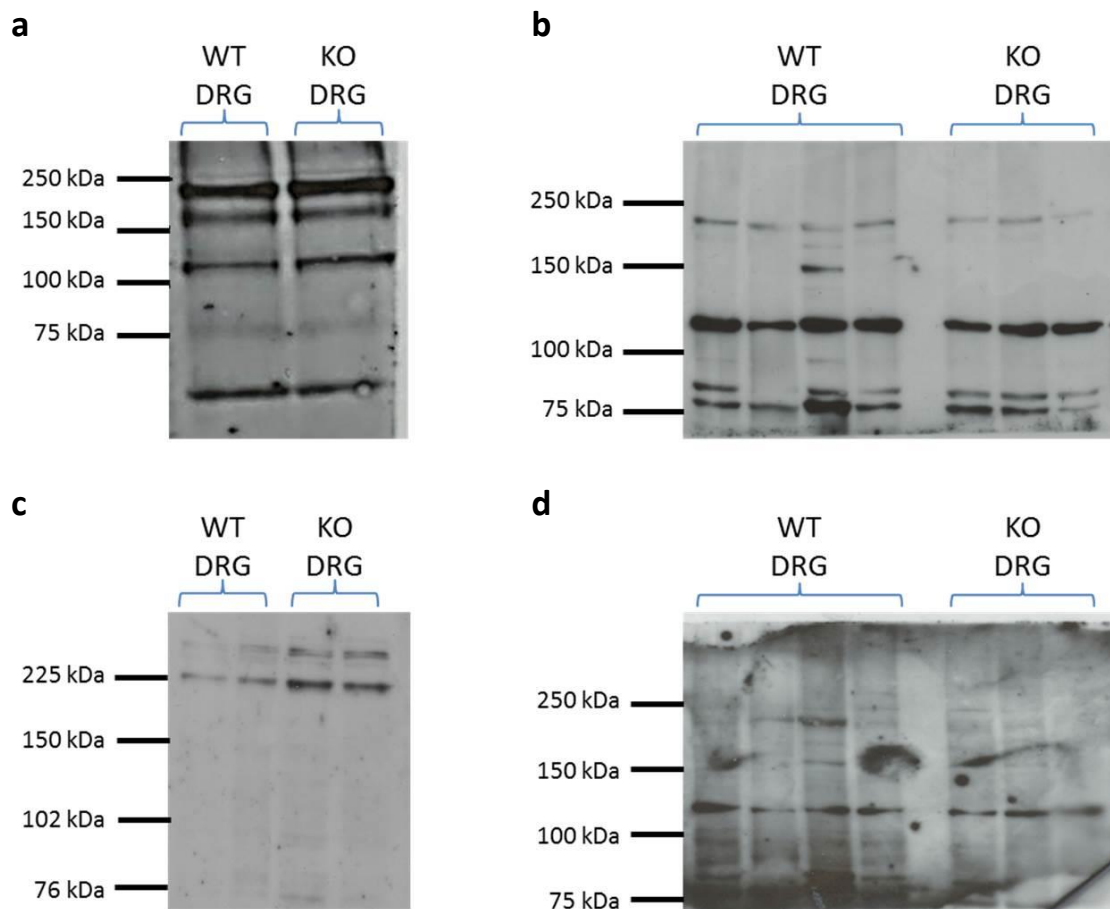


Figure 39. Representative immunoblots of dorsal root ganglia (DRG) lysates from TRPV1 WT and KO mice, using a number of different anti-TRPV1 antibodies. (a) ab4579, Abcam, Cambridge, UK. (b) ACC-030 Batch 2, Alomone Labs, Israel. (c) V2764, Sigma-Aldrich, UK. (d) anti-TRPV1 antibody synthesised in-house and kindly donated by Prof. Michael Caterina, Johns Hopkins, USA.

5.3.3 TRPV4, but not TRPV1, is active in vascular cells under basal conditions

Despite a lack of sufficiently selective antibodies for TRPV1, selective agonists for both TRPV1 and TRPV4 are commercially available. Consequently, we aimed to characterise TRPV1 and TRPV4 protein activity in cultured vascular cells, using selective agonists and the fluorescent Ca^{2+} dye Fura-2/AM to quantify changes in intracellular Ca^{2+} levels in response to channel activation. Given that microplate-based fluorometry provides an average reading across the entire surface of a well, a signal may remain undetected if only a small number of cells respond to a particular stimulus. In order to gain the most detailed picture of TRPV1 and TRPV4 activity in isolated vascular cells, therefore, we opted initially to conduct single-cell live imaging, using a fluorescent microscope-based system.

Freshly isolated murine pulmonary arterial endothelial cells (mPAEC) and murine aortic smooth muscle cells (mASMC) loaded with Fura-2/AM were perfused with HBSS for 30 sec in order to record basal intracellular calcium levels. Cells were then sequentially perfused with capsaicin (1 μM), GSK1016790A (GSK; 1 μM) and positive control (either 1 μM ionomycin or 100 μM adenosine triphosphate; ATP), with intermittent washing stages. A fluorescence ratio change of 0.1 or greater was deemed responsive to agonist stimulation (Alexander et al., 2013) and any cells non-responsive to positive control were excluded from analysis. Capsaicin did not appear to elicit any response in mPAEC (**Fig. 40a & c**), or mASMC (**Fig. 40b & d**), though cells responded robustly to TRPV4 activation, with approximately 60% response rate to GSK in both cell types (**Fig. 40**). The mean ratio change following TRPV4 activation in mPAEC was 0.2974 ± 0.053 (**Fig. 40g & h**).

Similarly, no evidence of capsaicin responsiveness was observed in primary endothelial cells isolated from bovine aortae: 2.65% of cells responded to capsaicin application, compared with 71.73% of cells responding to GSK (**Fig. 41**).

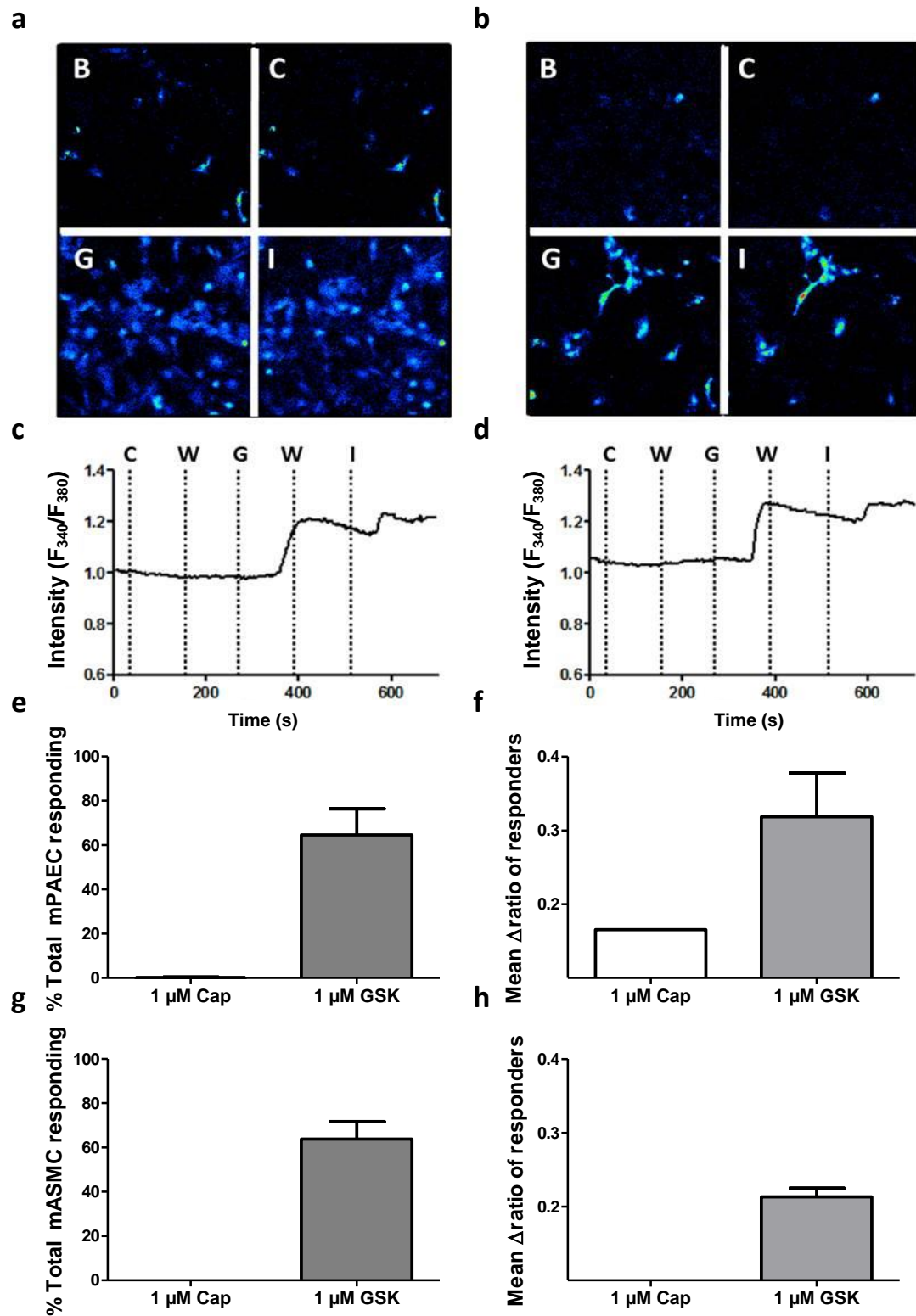


Figure 40. Basal TRPV1 and TRPV4 activity in murine vascular cells. (a) and (b) Representative fluorescent images of mPAEC and mASM, respectively, at baseline [B], and after stimulation with 1 μ M capsaicin [C], 1 μ M GSK1016790A [G] and 1 μ M ionomycin [I]. (c) and (d) Representative traces of ratiometric emission intensity following different treatments in mPAEC and mASM, respectively (C = 1 μ M capsaicin; W = wash; G = 1 μ M GSK1016790A; I = 1 μ M ionomycin). (e) and (f) Percentage of mPAEC and mASM, respectively, responding to 1 μ M capsaicin (Cap) and 1 μ M GSK1016790A (GSK). Data represent mean percentage of responders over 6 and 5 coverslips, respectively, \pm SEM (5 – 60 cells per coverslip). (g) and (h) Mean ratio change of responding mPAEC and mASM \pm SEM across 6 and 5 coverslips, respectively. A ratio change ≥ 0.1 was considered responsive.

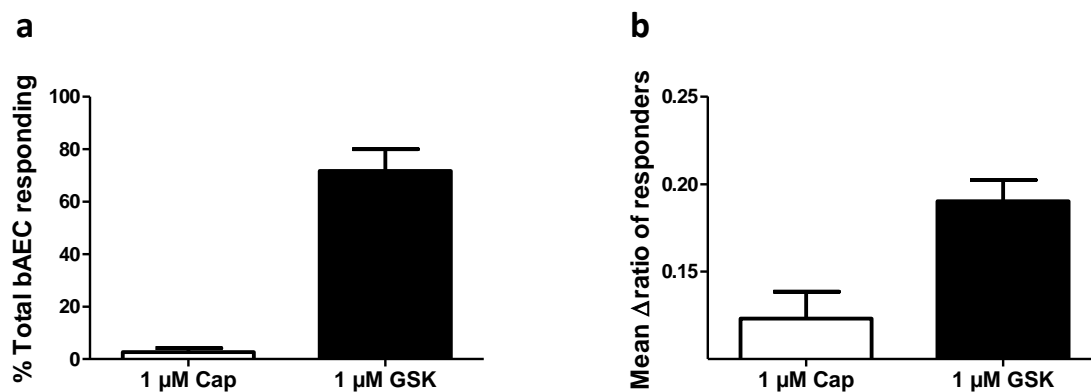


Figure 41. Basal TRPV1 and TRPV4 activity in bovine endothelial cells. (a) Percentage of bAEC responding to 1 μ M capsaicin (Cap) and 1 μ M GSK1016790A (GSK). Data represent mean percentage of responders \pm SEM over 4 coverslips (5 – 60 cells per coverslip). **(b)** Mean ratio change \pm SEM of responding bAEC across 4 coverslips. A ratio change ≥ 0.1 was considered responsive.

5.3.4 GSK1016790A responses are TRPV4-specific, and are optimal in low-glucose culture conditions

This study subsequently aimed to determine that the response to GSK1016790A was indeed mediated by TRPV4 channels, using the selective TRPV4 antagonist HC-067047 (Sigma-Aldrich, SML0143). Given that a response to GSK1016790A was observed in a large proportion of cells (approximately 60% in mPAEC and 70% in bAEC) a high-throughput microplate-based imaging system was used for further investigation.

For previous experiments, cells had been maintained in Dulbecco's modified Eagle's medium containing 4.5 g/L glucose (equivalent to approximately 25 mM). In 2012, a report was published demonstrating significant downregulation of TRPV4 in high-glucose (25 mM) conditions (Hills et al., 2012). Consequently, we used the microplate-based imaging system to characterise TRPV4 activity in bAEC maintained under three different culture conditions: high-glucose DMEM (4.5 g/L), low-glucose DMEM (1 g/L), or specialised Endothelial Cell Medium (1 g/L glucose).

A concentration-dependent response to GSK1016790A was observed on non-linear regression analysis, and the concentration-response curve for each culture condition was shifted towards the right following pre-treatment for 10 min with 1 μ M HC-067047, indicating competitive antagonism (**Fig. 42**). Maximum response (E_{max}) to GSK1016790A was roughly equivalent across culture conditions, though EC_{50} was lowest in low-glucose DMEM (**Table 11**). Consequently, low-glucose DMEM was used in all subsequent experiments.

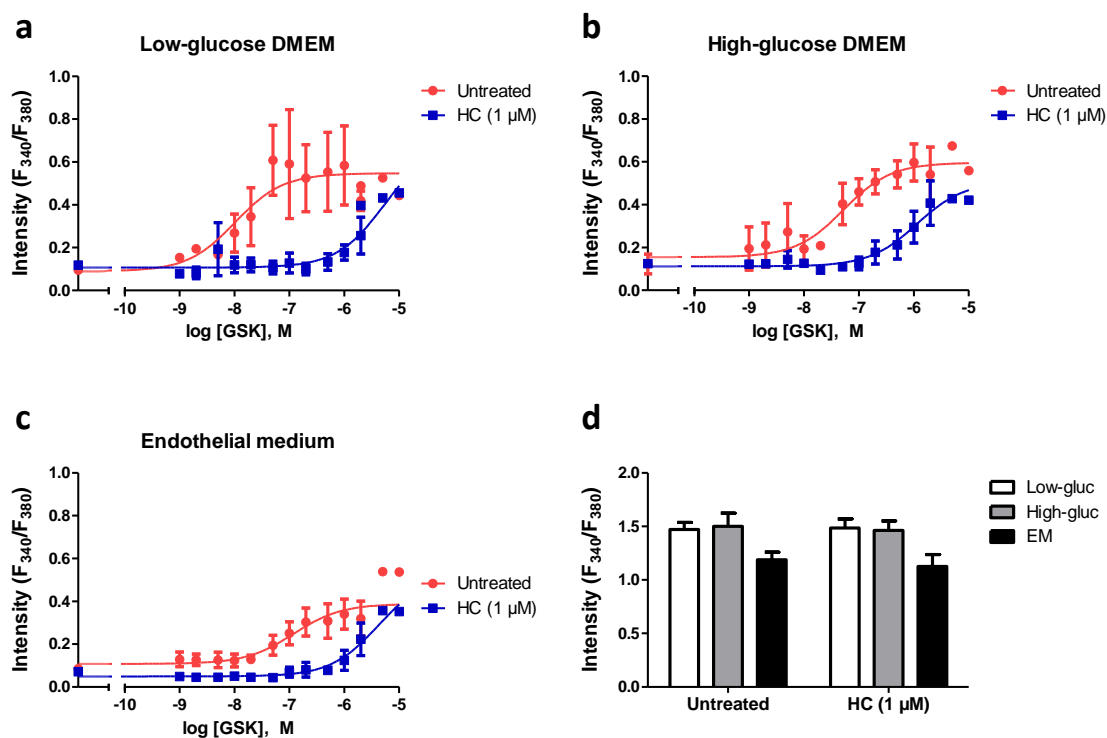


Figure 42. TRPV4 activity in bovine aortic endothelial cells under different culture conditions. Concentration-response curves were constructed for the TRPV4 agonist GSK1016790A (GSK) in the presence or absence of 1 μ M HC-067047 (HC; 10 min pre-treatment) in (a) low-glucose DMEM, (b) high-glucose DMEM and (c) specialised endothelial cell medium ($n = 4$). (d) Basal Ca^{2+} levels under different culture conditions. Low-gluc = low-glucose DMEM; high-gluc = high-glucose DMEM; EM = endothelial medium.

	Low-glucose (1 g/mL)		High-glucose (4.5 g/mL)		Endothelial medium (1 g/mL)	
	Control	HC-067047 (1 µM)	Control	HC-067047 (1 µM)	Control	HC-067047 (1 µM)
EC₅₀	10.25 nM (1.44–73.11 nM)	5.30 µM (1.32–21.38 µM)	52.12 nM (18.15–149.97 nM)	1.15 µM (0.37–3.56 µM)	118.58 nM (32.81–429.54 nM)	4.14 µM (1.50–11.46 µM)
E_{max}	0.547 (0.439–0.655)	0.692 (0.243–1.140)	0.596 (0.515–0.678)	0.509 (0.335–0.682)	0.389 (0.309–0.468)	0.525 (0.276–0.773)

Table 11. TRPV4 activity in bovine aortic endothelial cells under different culture conditions. Data are presented as mean (95% CI).

5.3.5 LPS may sensitise TRPV4 in endothelial cells

In order to characterise the changes in TRPV4 activity under inflammatory conditions more extensively, we used a high-throughput microplate-based system to monitor the time- and concentration-dependent effects of LPS on TRPV4-induced Ca^{2+} release. Cells were stimulated with 100 nM GSK1016790A, as this concentration was found to produce a reliable Ca^{2+} signal in this system. While responses to GSK1016790A were not altered with increasing concentrations of LPS, or with increasing exposure time (1–24 h), responses to ATP declined in a concentration- and time-dependent manner, indicative of cytotoxicity (**Fig. 43a–c**). When responses to GSK1016790A were normalised to positive control, activity appeared to increase with increasing concentrations of LPS, following 24-h exposure (**Fig. 43d–f**). 24-h exposure to LPS was correspondingly found by MTT assay to decrease cell viability in a concentration-dependent manner, though increased TRPV4 activity did not appear to be involved in this cytopathic response: co-incubation of bAEC with 10 μM HC-067047 and LPS did not improve cell viability (**Fig.44**).

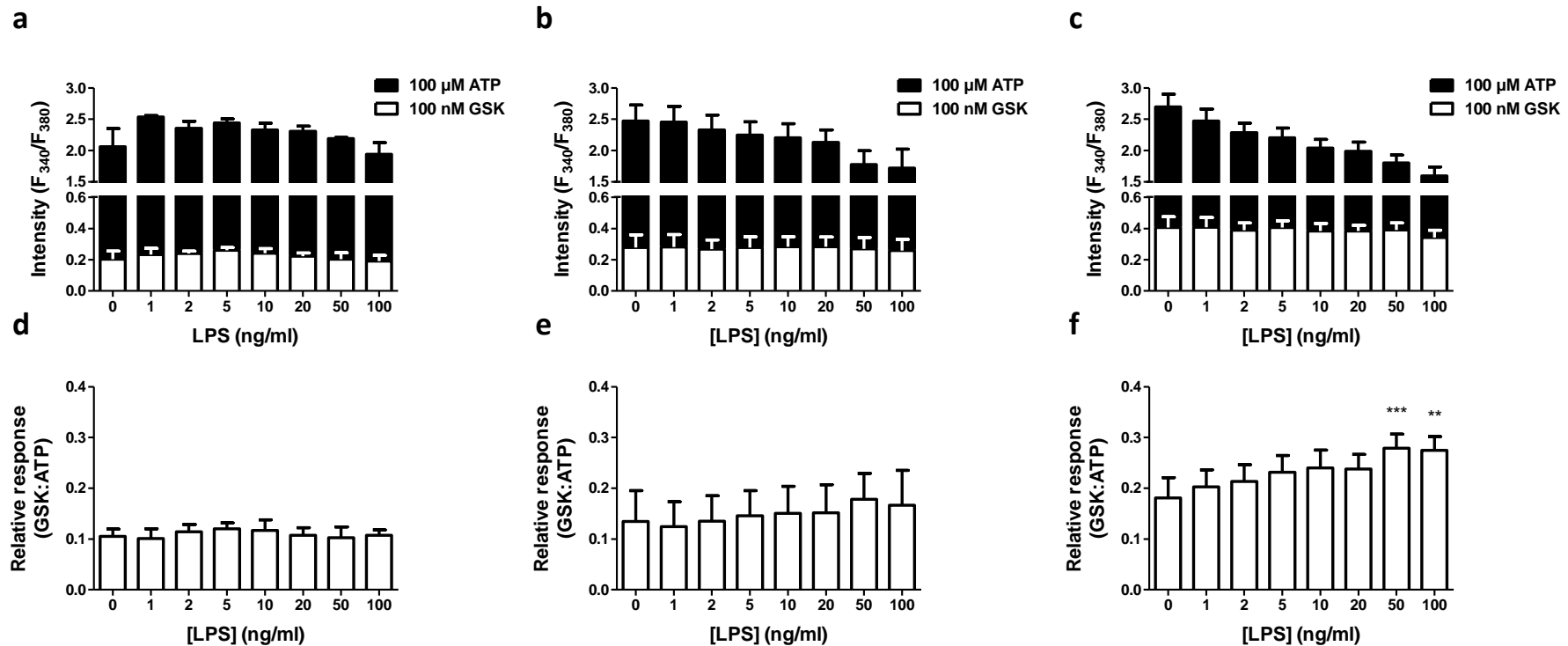


Figure 43. TRPV4 activity in bovine aortic endothelial cells pre-treated with LPS. (a–c) Responses to 100 nM GSK1016790A (GSK) or positive control (100 μ M ATP) following exposure to increasing concentrations of LPS for 1 h, 6 h or 24 h, respectively. (d–f) Responses to 100 nM GSK1016790A normalised to ATP responses following exposure to increasing concentrations of LPS for 1 h, 6 h or 24 h, respectively. Data are presented as mean \pm SEM (n = 7). **p<0.01, ***p<0.001 relative to untreated controls, 1-way ANOVA + Bonferroni post-hoc test.

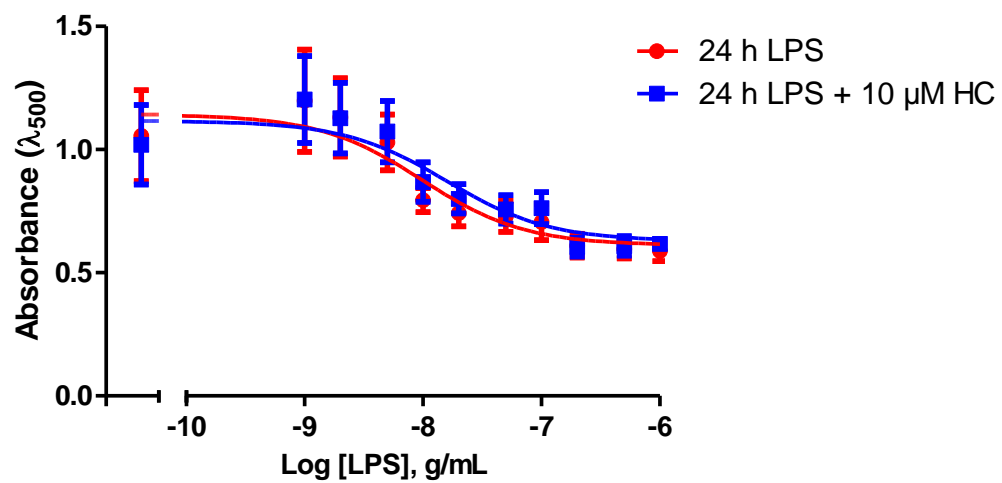


Figure 44. Bovine aortic endothelial cell viability following 24-h exposure to increasing concentrations of LPS. Cell viability was assessed by MTT assay: decreased formazan production, and corresponding decreased absorbance at 560 nm indicate decreased viability. Data are presented as mean \pm SEM, $n = 7$.

5.3.6 TRPV4, but not TRPV1, is expressed in murine bone marrow-derived macrophages

Based on previous reports that TRPV1 expressed in peritoneal macrophages could influence sepsis pathogenesis (Fernandes et al., 2012), and that alveolar macrophage-derived TRPV4 may contribute to lung dysfunction (Alvarez et al., 2006, Hamanaka et al., 2010), we aimed to determine whether TRPV1 and TRPV4 were expressed in bone marrow-derived macrophages (BMDM) isolated from the femur bones of TRPV4 KO and WT mice. GSK1016790A caused a concentration-dependent increase in intracellular Ca^{2+} levels in WT, but not in TRPV4 KO mice (**Fig. 45**). Capsaicin, on the other hand, did not cause a significant rise in intracellular Ca^{2+} , suggesting that TRPV1 is not present in this cell type.

We also aimed to determine the functional significance of TRPV4 expression in BMDM, by measuring phagocytosis of fluorescent latex beads in the presence and absence of LPS and GSK1016790A. While LPS increased bead uptake by BMDM, this was not affected by TRPV4 stimulation (**Fig. 46**).

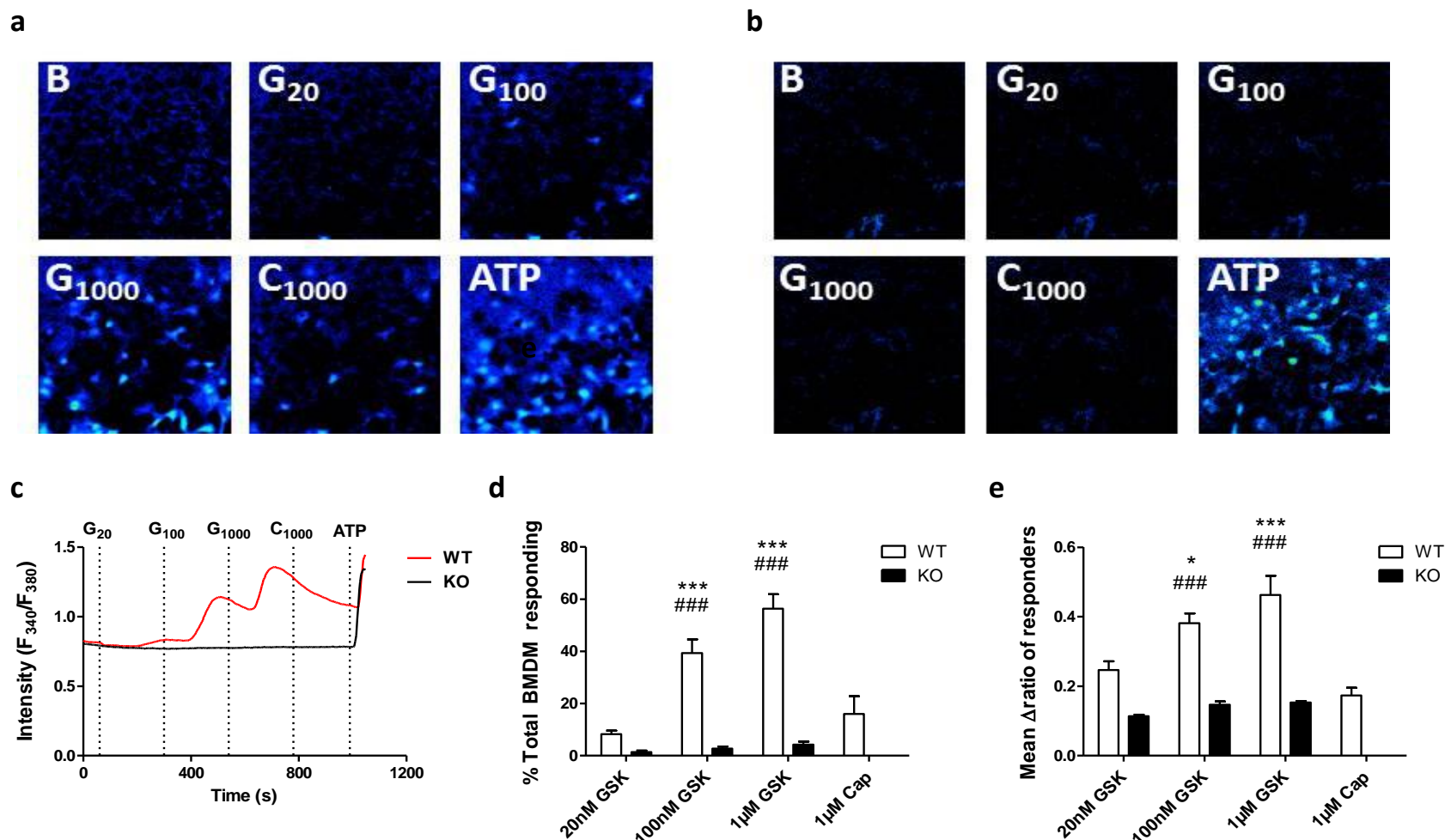


Figure 45. Basal TRPV1 and TRPV4 activity in murine BMDM. (a) and (b) Representative fluorescent images of BMDM from TRPV4 WT and KO mice, respectively, at baseline [B], and after stimulation with 20 nM GSK1016790A [G₂₀], 100 nM GSK1016790A [G₁₀₀], 1 μM GSK1016790A [G₁₀₀₀], 1 μM capsaicin [C₁₀₀₀] and 100 μM ATP [ATP]. (c) Representative traces of ratiometric emission intensity following different treatments in BMDM. (d) Percentage of BMDM responding to increasing concentrations of GSK1016790A (GSK) and 1 μM capsaicin (Cap). Data represent mean percentage of responders over 7 coverslips ± SEM (80–100 cells per coverslip). (e) and (f) Mean ratio change of responding BMDM ± SEM across 7 coverslips. The threshold for response was 0.1. **p*<0.05, ***p*<0.001 relative to 20 nM GSK, ###*p*<0.001 relative to KO counterparts, 1-way ANOVA + Bonferroni post-hoc test.

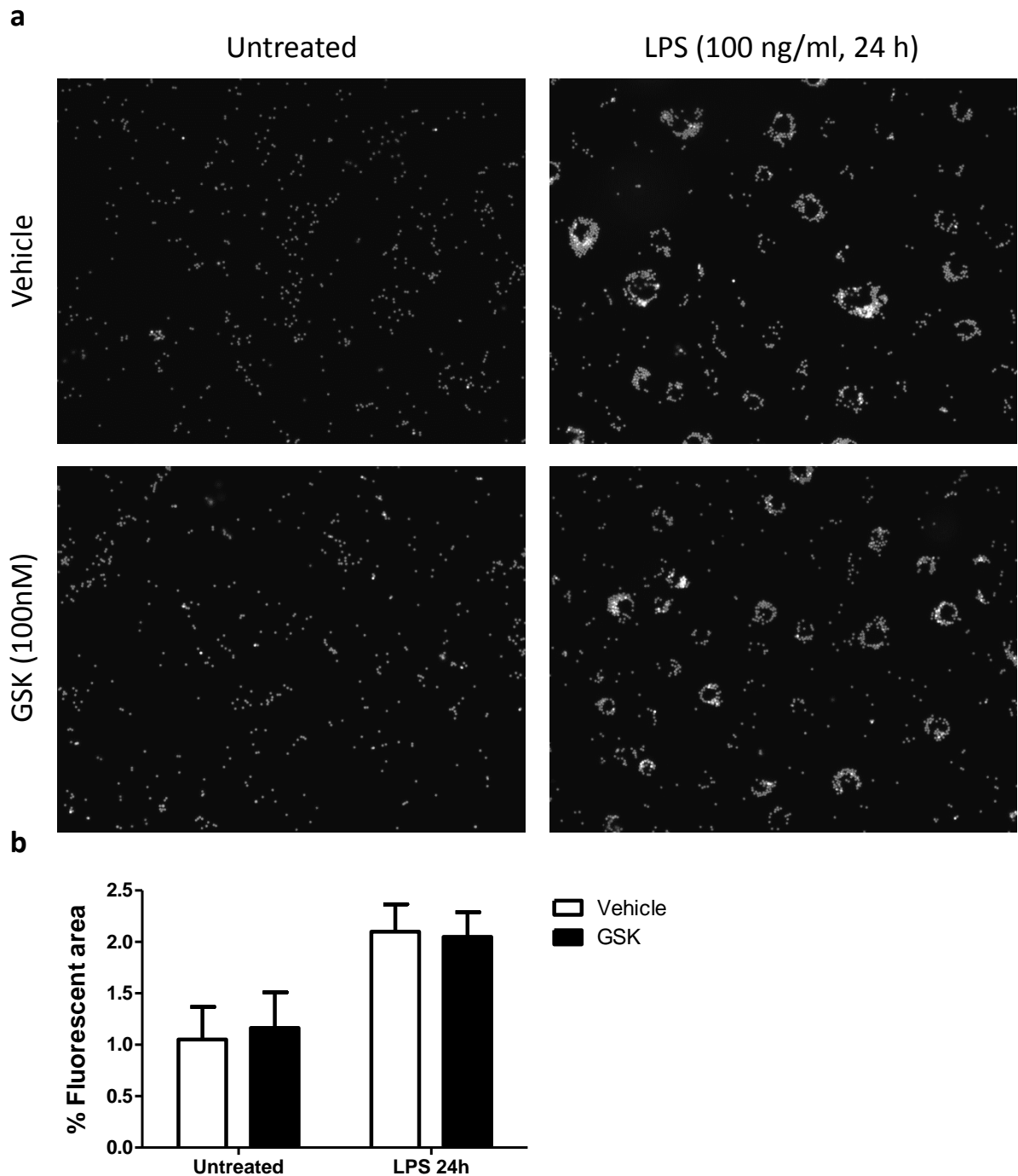


Figure 46. Phagocytosis of fluorescent latex beads by murine BMDM. (a) Representative fluorescent images of BMDM either untreated or following stimulation with LPS (100 ng/ml, 24 h) in the presence of either GSK1016790A (100 nM; GSK) or vehicle (2% DMSO in DMEM). (b) Percentage of total area with fluorescence, as quantified by ImageJ software. Data are presented as mean \pm SEM (n = 8 chambers from 2 mice).

5.4 Summary

The key findings from this study are as follows:

1. TRPV1 is expressed at an mRNA level in vascular endothelial cells, but does not appear to be expressed as an active protein in the cell lines tested, either under basal or inflammatory conditions
2. Commercially available anti-TRPV1 antibodies do not appear to be selective for TRPV1
3. TRPV4 is expressed and functional in endothelial cells, and may be sensitised under inflammatory conditions
4. TRPV4 does not appear to contribute to LPS-induced cytotoxicity in endothelial cells
5. TRPV4, but not TRPV1, is expressed in murine BMDM, but its activation does not appear to influence phagocytic function

5.5 Discussion

Following numerous reports suggesting that TRPV1 and TRPV4 may be important endothelial regulators, we aimed to characterise their expression and activity in a number of different endothelial cell lines. Functional expression of TRPV4 in the vascular endothelium is widely accepted (Baylie and Brayden, 2011), and data obtained in this study is consistent with published literature. While endothelial expression of TRPV4 has been demonstrated convincingly in a number of physiological settings, however, its role in pathological contexts has not yet been explored.

The role – and indeed the very presence – of TRPV1 in endothelial cells, on the other hand, remains highly contentious. While several groups have reported evidence of endothelial TRPV1 expression (Fantozzi et al., 2003, Yang et al., 2010a, Bratz et al., 2008), others have failed to reproduce these findings (Marrelli et al., 2007, Cavanaugh et al., 2011). Many studies have relied on mRNA expression alone, and protein quantification has often been conducted in the absence of appropriate controls, or using antibodies that have not been validated in TRPV1 KO tissue. We aimed to clarify these discrepancies using a combination of biochemical and functional analysis in a number of different endothelial cell lines.

5.5.1 TRPV1 is expressed at an mRNA but not a protein level in cultured endothelial cells

Initial experiments were aimed at determining expression levels of TRPV1 in vascular cells and tissues. Using reverse transcription and PCR amplification of vascular cDNA, we found evidence of TRPV1 mRNA expression in aortic lysates of WT, but not KO mice, as well as in freshly isolated and immortalised endothelial cells from three different species. Although we initially intended to compare tissue protein levels of TRPV1 between naïve and septic animals, we were unfortunately unable to do this by traditional antibody-recognition methods, since the commercially available antibodies tested were found to be insufficiently selective for TRPV1.

ACC-030 (Alomone Labs) is a widely used anti-TRPV1 antibody, that in previous reports showed no immunoreactivity in samples from TRPV1 KO mice (albeit in the absence of a protein loading control) (Yang et al., 2010b). We observed clear and distinct bands in aortic and DRG lysates from KO mice of identical origins, suggesting that this antibody is an unsuitable indicator of TRPV1 protein expression. Furthermore, these bands were observed at approximately 75 kDa – 20kDa smaller than the predicted molecular weight of TRPV1 (Caterina et al., 1997): further evidence of non-selective immunoreactivity. While the other antibodies tested are similarly widely used, they do not appear to have been validated in TRPV1 KO samples, and according to our results are likewise insufficiently selective for TRPV1 protein.

Although TRPV1 KO mice are functional knockouts, retaining the C-terminus of the TRPV1 gene against which most TRPV1 antibodies are raised, our sequencing data demonstrate the presence of a TAG stop codon inside the inserted neomycin cassette, precluding the possibility of residual C-terminal translation. These observations call into question much of the data on TRPV1 expression that has been published to date, and suggest that numerous reports of TRPV1 protein expression will require considerable further validation.

In the absence of a biochemical method for determining TRPV1 protein levels, we attempted to assess TRPV1 expression and activity by functional assay, using the highly selective agonist capsaicin. Freshly isolated murine and bovine endothelial cells were perfused with 1 μ M capsaicin, roughly equivalent to the EC_{50} of rat TRPV1 in heterologous expression systems (Caterina et al., 1997, Welch et al., 2000). Neither cell type exhibited any calcium influx in response to capsaicin treatment, despite responding robustly to TRPV4 activation and positive controls.

Although cellular yield was insufficient to perform molecular analysis of primary murine endothelial cells, TRPV1 mRNA was detected in other vascular tissues and cells, including immortalised murine endothelial cells, and we were surprised, therefore, that no evidence of functional TRPV1 expression was observed here, particularly given that a previous group reported significant Ca^{2+} influx in murine aortic endothelial cells in response to 1 μ M capsaicin (Yang et al., 2010b). It is possible that regionally restricted expression or species differences could account for this discrepancy, or that the culture conditions used could have contributed to downregulation of the channel. Furthermore, despite finding no evidence of functional TRPV1 expression in the endothelium of two different vascular beds (aorta and lung) from two different species (cow and mouse, respectively), it is possible that a specific stimulus (perhaps inflammatory) is required in order for TRPV1 translation or membrane insertion to occur. It is notable, however, that TRPV1 mRNA expression has also been observed in odontoblasts, which similarly do not appear to respond to capsaicin (Egbuniwe et al., 2014).

Given that TRPV1 KO mice appear to have a normal haemodynamic profile under basal conditions (Pacher et al., 2004, Marshall et al., 2013), it is possible that protein translation and activity in vascular tissue are minimal in a physiological setting, and that under pathological conditions the receptor is upregulated, as observed previously in other cell types (Stander et al., 2004). A scenario in which endothelial cells retain pools of TRPV1 mRNA that is translated under conditions of stress and inflammation, may explain our observations of clear TRPV1 mRNA expression with despite no evidence of receptor activity. Alternatively, TRPV1 protein may be retained in intracellular pools, either in the endoplasmic reticulum or in cytoplasmic

vesicles, in endothelial cells, as described previously (Guo et al., 1999, Morenilla-Palao et al., 2004)

While pre-treatment of bovine endothelial cells with the bacterial endotoxin LPS did not induce responsiveness to capsaicin in this study, it is possible that a different inflammatory stimulus would have increased activity. Alternatively, while TRPV1 may be expressed at an mRNA level in all endothelial tissue, it may only be functional in microvascular beds. Previous studies have demonstrated TRPV1 mRNA in human pulmonary capillary endothelial cells, which respond to the capsaicin analogue nonivamide and are sensitised by LPS (Thomas et al., 2012). Given that TRPV1 KO mice exhibit microvascular dysfunction (**Chapter 4**), we acknowledge that bovine aortic endothelial cells do not represent the most relevant model for *in vitro* assessment of vascular TRPV1 activity. Numerous proteins, including selectins and cell adhesion molecules, for example, show widely divergent expression patterns across the vascular system (Aird, 2012); it is conceivable therefore, that expression of TRPV1 in different endothelial layers may be similarly heterogeneous. While it was not possible to isolate microvascular cells in this study (isolates from murine lungs would have contained a mix of macrovascular and microvascular endothelial cells, and yield was furthermore prohibitively low), further research using pure populations of microvascular cells could shed further light on the endothelial expression of TRPV1 under basal and inflammatory conditions.

5.5.2 Cultured endothelial cells response robustly to TRPV4 activation, and may become sensitised to TRPV4 stimuli under inflammatory conditions

Consistent with previous reports (Baylie and Brayden, 2011), functional endothelial TRPV4 expression was demonstrated in this study in a number of different cell types. The robust response to the TRPV4 agonist GSK1016790A was antagonised by HC-067047, suggesting selective TRPV4 activation. The cellular response to GSK1016790A was found to be relatively delayed with a slow wash-off rate. This may be indicative of an intracellular binding site for the agonist. Previous studies using phorbol esters, such as 4 α -phorbol 12,13-didecanoate (4 α -PDD), to activate TRPV4 in transfected HEK cells and isolated murine aortic ECs showed similarly slow kinetics of activation and dissociation (Watanabe et al., 2002a). It is possible that the synthetic agonist GSK1016790A, like 4 α -PDD, must first diffuse across the cellular membrane in order to access an intracellular binding site, accounting for the notable lag between administration of the GSK1016790A compound and an increase in intracellular [Ca²⁺].

While the role of TRPV4 in endothelial function has been studied widely, its activity under pathological conditions has not been characterised. Recent reports have suggested that excessive TRPV4 activation causes endothelial failure and circulatory collapse (Willette et al.,

2008). Given that numerous modulators of TRPV4 – arachidonic acid metabolites (Watanabe et al., 2003b), shear stress (Kohler et al., 2006, Hartmannsgruber et al., 2007), osmolality (Strotmann et al., 2000, Liedtke et al., 2000) – are altered in sepsis, we aimed to characterise TRPV4 activity under inflammatory conditions.

Using a high-throughput microplate-based system, intracellular $[Ca^{2+}]$ in response to GSK1016790A was quantified following pre-incubation with increasing concentrations of LPS. While the overall response to 100 nM GSK1016790A was relatively consistent with increasing LPS concentrations and exposure time, responsiveness to ATP – the positive control – was markedly reduced in a concentration-dependent manner after 24-h exposure to LPS. While it is possible that purinergic 2X (P2X) receptors – thought to be chiefly responsible for mediating ATP-induced effects in the endothelium (Harrington et al., 2007) – are simply downregulated following LPS exposure, we were unable to find any previous reports of LPS-induced desensitisation of ATP responsiveness. On the contrary, responsiveness to ATP appears to be increased in other cell types under inflammatory conditions (Xu and Huang, 2002), and several receptor subtypes are upregulated in cultured human umbilical vein endothelial cells stimulated with LPS, tumour necrosis factor (TNF)- α , interferon- γ (IFN- γ) or interleukin-1 β (IL-1 β) (Wilson et al., 2007). We therefore considered the reduced responsiveness to ATP in our system to be representative of increased cell death, and consequently normalised GSK1016790A responses to ATP responses. Following normalisation, a concentration-dependent sensitisation of endothelial cells to GSK1016790A was observed, suggesting that endothelial TRPV4 activity could be increased in sepsis. Further experiments using a receptor-independent positive control, such as ionomycin, would be required to confirm these observations, however.

Consistent with this interpretation of decreased ATP responsiveness, 24-h exposure to LPS was found to cause a concentration-dependent decrease in endothelial cell viability. This likely contributes to the vascular dysfunction that characterises sepsis. While a previous study has shown that excessive TRPV4 activation causes endothelial detachment and necrosis (Willette et al., 2008), incubation of bAEC with HC-067047 did not attenuate LPS-induced loss of cell viability. This suggests that tonic activation of TRPV4 in the presence of LPS does not contribute to cytotoxicity in this system, and that exogenous or more potent agonism is required to induce endothelial death. Further experiments, in which endothelial viability is assessed in the presence of both LPS and GSK1016790A may be informative. While endothelial synthesis of endogenous agonists of TRPV4, such as anandamide (Watanabe et al., 2003b), may be increased under inflammatory conditions (Opitz et al., 2007), this did not appear to play a role in cell death in our culture conditions; if any endogenous autocrine TRPV4

activation was present in this system, it was likely substantially lower than the level of GSK1016790A-induced TRPV4 activation that caused endothelial cell death in previous studies (Willette et al., 2008).

5.5.3 TRPV4, but not TRPV1, is present in bone marrow-derived macrophages, but does not appear to regulate phagocytosis

Previous reports have suggested that TRPV1 (Fernandes et al., 2012) and TRPV4 (Alvarez et al., 2006, Hamanaka et al., 2010) are expressed in macrophages; given the known link between Ca^{2+} influx and phagocytosis (Diler et al., 2014), expression of these two Ca^{2+} -permeable channels in this cell type may indicate a potential role in phagocytosis. In view of the central role of macrophages in sepsis (Warren, 2011), we aimed to characterise TRPV1 and TRPV4 expression in murine bone marrow-derived macrophages. While GSK1016790A caused a robust and concentration-dependent increase in intracellular $[\text{Ca}^{2+}]$ in BMDM from WT but not TRPV4 KO mice, only very few cells responded to capsaicin, and with a comparatively low response size. Owing to the very slow wash-off rate of GSK1016790A, it is possible that any responses recorded after stimulation with capsaicin were actually the result of residual TRPV4 activity. While the perfusion sequence was not randomised in this study – a limitation that should be addressed in future experiments – the very small number of responding cells, and the very low response size following capsaicin stimulation, suggest that significant expression of TRPV1 is not present in this cell type under the culture conditions used.

These data are inconsistent with a previous report that TRPV1 plays a key role in macrophage phagocytosis (Fernandes et al., 2012). There are numerous potential explanations for this discrepancy. Firstly, the role of TRPV1 may differ between different macrophage populations: only quiescent bone marrow-derived macrophages were used in this study, whereas Fernandes and colleagues characterised TRPV1 activity in peripheral macrophages isolated from the peritoneal cavity of TRPV1 WT and KO mice, which may have had a different activation state. Secondly, while macrophages from TRPV1 WT and KO mice appeared to function differently (Fernandes et al., 2012), the expression of TRPV1 in WT macrophages was only established at the mRNA level; indirect regulation of macrophage function by TRPV1-dependent mechanisms may also have accounted for the divergent behaviour of WT and KO cells. Finally, it is of note that in the study by Fernandes and colleagues, LPS stimulation did not increase phagocytosis of latex beads; somewhat confusingly, however, the phagocytic capacity of macrophages isolated from TRPV1 KO mice appeared to be reduced below baseline levels in the presence of LPS, suggesting that LPS inhibits phagocytosis in the absence of TRPV1. While this observation was not explained by the authors, it is inconsistent with previous reports that the phagocytic activity of both bone marrow-derived macrophages (Cooper et al., 1984) and

peritoneal macrophages (Anand et al., 2007) is potentiated by LPS. The relatively high concentration of latex beads used by Fernandes and colleagues, combined with the long incubation time of 24-h, may have induced maximum bead uptake that could not be further potentiated by LPS.

Using a 10-fold lower concentration of beads, and an incubation time of 1 h, we observed increased bead uptake by BMDM in the presence of LPS, consistent with previous reports (Cooper et al., 1984, Anand et al., 2007). Co-incubation with GSK1016790A, however, did not influence bead uptake, suggesting that TRPV4 does not contribute to phagocytic function in the culture conditions used.

While prior reports have suggested that TRPV4 regulates alveolar macrophage activation (Alvarez et al., 2006, Hamanaka et al., 2010), neither previous study evaluated phagocytic function. In addition to phagocytosis, macrophages also participate in the production of cytokines, chemokines and reactive oxygen species; it is possible that expression of TRPV4 in this cell type is related to one of these latter functions, rather than to phagocytosis. While we observed no change in phagocytic function in the presence of a TRPV4 agonist, however, our data do not preclude completely the potential involvement of the channel in this process. Further investigation using a larger sample size, a wide range of agonist concentrations, and selective antagonism to investigate any tonic activity in this cell type will elucidate the role of TRPV4 in phagocytosis further. Furthermore, we used a relatively crude measure of uptake by counting fluorescent pixels. Fluorescent staining and analysis of overlap between TRPV4 expression, macrophage markers and phagocytosed beads would help to elucidate whether subsets of TRPV4-expressing macrophages do have a specific role in pathogen phagocytosis.

5.5.4 Concluding remarks

Despite demonstrating robust TRPV1 mRNA expression in aortic lysates and isolated endothelial cells, we have found no evidence of functional TRPV1 protein expression in these tissues, in spite of numerous previous reports to the contrary. On the other hand, these data do not preclude the possibility of induced endothelial TRPV1 expression under certain pathological conditions, or indeed of functional expression in specific subpopulations of endothelial cells (from the microvasculature, for example).

We have observed convincing evidence of functional TRPV4 expression in the vascular endothelium of three separate species, and in murine macrophages, consistent with previous reports and indicative of an important role for TRPV4 in these cells. We have observed a sensitisation of the channel under inflammatory conditions, and given recent reports linking

excessive TRPV4 activation to profound hypotension and circulatory collapse (Willette et al., 2008), we hypothesise that TRPV4 may be an important vascular regulator in sepsis.

CHAPTER 6 | THE ROLE OF TRPV4 IN SEPSIS-ASSOCIATED CARDIOVASCULAR DYSFUNCTION

6.1 Introduction

Transient receptor potential vanilloid 4 (TRPV4) was discovered by two different groups simultaneously (Liedtke et al., 2000, Strotmann et al., 2000), following a database screen for sequences with similarity to TRPV1, TRPV2, and the *C. elegans* TRPV isoform OSM-9. Due to an initial lack of consensus nomenclature, TRPV4 was variously called OTRPC4 (Strotmann et al., 2000), VR-OAC (Liedtke et al., 2000), TRP12 (Wissenbach et al., 2000) and VRL-2 (Delany et al., 2001) by the different groups to clone the receptor. Originally identified as an osmosensor in the kidney (Strotmann et al., 2000, Liedtke et al., 2000), TRPV4 is expressed in numerous tissues, and has been implicated in a wide variety of physiological processes, including vascular regulation.

6.1.1 TRPV4 structure

TRPV4 is an 871-amino-acid protein that shares numerous structural features with TRPV1: the channel has large intracellular N- and C-termini, six transmembrane domains with a re-entrant pore-forming loop between domains 5 and 6, and at least three ankyrin repeat domains in the cytosolic N-terminus (Plant and Strotmann, 2007). Despite sharing a high degree of sequence homology with TRPV1 (among other TRPV channels), TRPV4 was originally thought to exist exclusively as a homotetramer (Hellwig et al., 2005), though there have been recent reports of heterotetramerisation with TRPC1 and TRPP2 in vascular endothelial cells and renal cortical collecting duct cells (Du et al., 2012, Ma et al., 2010). The structure of the TRPV4 subunit is shown schematically in **Fig. 47**.

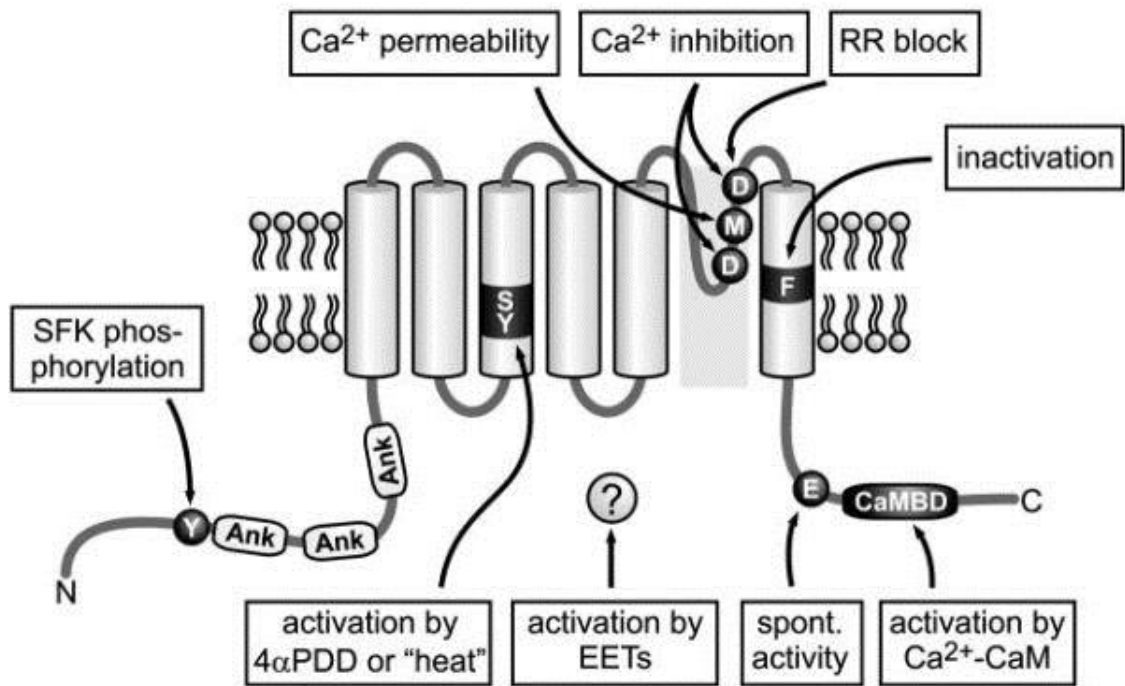


Figure 47. Structural features of the TRPV4 subunit. TRPV4 subunits comprise 6 transmembrane domains with a re-entrant pore-forming loop between domains 5 and 6, and cytosolic N- and C-termini. Residues important for channel function and regulation are indicated in black shaded areas. Three ankyrin repeat domains and a SRC family kinase (SFK) phosphorylation site are present in the N-terminus. Serine and tyrosine residues in transmembrane domain 3 are essential for channel activation by phorbol ester derivatives and heat. Residues in the pore-forming loop are essential for Ca²⁺ permeability, Ca²⁺-mediated channel inhibition, and inhibition by ruthenium red (RR). The C-terminus contains a putative binding site for Ca²⁺-calmodulin, as well as a residue essential for spontaneous channel activity. The mechanism of activation by epoxyeicosatrienoic acids (EETs) is unknown. Figure adapted from *TRP Ion Channel Function in Sensory Transduction and Cellular Signaling Cascades*, Chapter 9 (Plant and Strotmann, 2007).

6.1.3 Tissue expression

TRPV4 was originally identified in the kidney, and orthologues for numerous other species, including mice and humans, have been cloned from this tissue (Strotmann et al., 2000, Liedtke et al., 2000, Wissenbach et al., 2000, Delany et al., 2001). It is additionally expressed in a wide variety of other tissues, including the vascular endothelium and smooth muscle, heart, lung, spleen, cochlea, liver, testes, placenta, trachea, salivary glands, adipose tissue and numerous brain regions (Filosa et al., 2013).

6.1.4 Channel activation

TRPV4 channels often exhibit spontaneous activity in heterologous expression systems, leading to elevated basal intracellular Ca^{2+} levels (Plant and Strotmann, 2007). This spontaneous activity is likely supported by as yet unidentified cellular factors, since currents disappear in ruptured patch recordings (Strotmann et al., 2000). Numerous endogenous and exogenous agonists have been identified for TRPV4, however.

6.1.4.1 Extracellular osmolarity

Originally identified as an osmosensor in the kidney (Strotmann et al., 2000, Liedtke et al., 2000), TRPV4 is highly sensitive to changes in extracellular osmolarity, responding to fluctuations as small as 1%; decreases in osmolarity result in membrane currents and a rise in intracellular $[\text{Ca}^{2+}]$, whereas increases above 300 mosmol/L inhibit membrane currents and decrease intracellular $[\text{Ca}^{2+}]$ in spontaneously active cells (Strotmann et al., 2000).

Responses to changes in osmolarity are temperature-dependent, and require a relatively intact intracellular environment: current responses to hypotonic solutions are significantly greater in perforated patch recordings than in ruptured patch recordings (Strotmann et al., 2000). The observation that TRPV4 still responds to other agonists – such as phorbol esters – in ruptured patch recordings (Watanabe et al., 2002a), suggests that channel activation by hypotonic stimuli relies on the generation of second messengers. Consistent with *in vitro* data demonstrating the osmosensitivity of heterologously expressed TRPV4, TRPV4 knockout (KO) mice exhibit osmoregulatory defects (Liedtke and Friedman, 2003, Mizuno et al., 2003).

6.1.4.2 TRPV4 as a mechanosensor

Because changes in extracellular osmolarity are associated with cell swelling and membrane stretch, numerous studies have investigated the possibility that TRPV4 is a mechanosensor. This notion is supported by the expression profile of TRPV4 in mechanosensory tissues, including the inner ear, vascular endothelium and sensory neurons. These investigations have

produced conflicting results, however. While initial experiments provided no evidence of TRPV4 activation in response to membrane stretch (Strotmann et al., 2000), a subsequent study recorded TRPV4 responses to cell inflation (Suzuki et al., 2003). Gao and colleagues also described an increase in intracellular $[Ca^{2+}]$ in TRPV4-expressing HEK293 cells exposed to shear stress that was sensitive to ruthenium red (a non-selective blocker of TRPV channels). This response was abolished when the temperature was reduced from 37°C to RT (Gao et al., 2003), suggesting that TRPV4 responses to mechanical stimuli, like hypotonic stimuli, are temperature-dependent. Two more recent studies also support the notion of direct TRPV4 activation by mechanical stimulation (Loukin et al., 2010, Matthews et al., 2010)

Whether a conserved mechanism underlies TRPV4 activation by hypotonic and mechanical stimulation is unclear. The observation of a markedly delayed response (≥ 20 s) to changes in osmolarity suggests that TRPV4 is not directly activated by this stimulus, and that cell swelling or initiation of downstream signalling pathways is required for activity. Whether mechanical stimulation of TRPV4 activates the channel directly, as proposed previously (O'Neil and Heller, 2005) is also not clear: while 3 ankyrin repeats in the N-terminus appear to anchor the receptor to the cytoskeleton (Xu et al., 2003), deletion of these ankyrin repeats does not abolish TRPV4 responses to hypotonicity (Liedtke et al., 2000).

6.1.4.3 Arachidonic acid metabolites

One potential pathway through which mechanical stimuli may activate TRPV4 involves the generation of arachidonic acid metabolites. Both the endocannabinoid anandamide and its downstream metabolite arachidonic acid have been shown to activate TRPV4 in vascular endothelial cells in a manner that is strongly inhibited by blockers of Cytochrome P450 (CYP450) epoxygenase (Watanabe et al., 2003b). CYP450 epoxygenase-mediated metabolism of arachidonic acid generates epoxyeicosatrienoic acids (EETs), which have been shown to be responsible for channel activation (Watanabe et al., 2003b, Vriens et al., 2004).

Responses to hypotonic stimuli are similarly blocked by structurally unrelated inhibitors of phospholipase A_2 (PLA₂; the enzyme responsible for release of arachidonic acid from phospholipid bilayers) and CYP450 epoxygenase (Vriens et al., 2004, Vriens et al., 2005). Correspondingly, activation of TRPV4 by hypotonic stimuli is potentiated by upregulation of CYP450 epoxygenase (Vriens et al., 2005). These data suggest that activation of TRPV4 by changes in extracellular osmolarity involves initiation of an intracellular signalling cascade, as described in **Fig. 48**. Constitutive activation of PLA₂ under isotonic conditions was originally

postulated to underlie spontaneous TRPV4 activity, though experiments using inhibitors of PLA₂ do not support this hypothesis (Vriens et al., 2004).

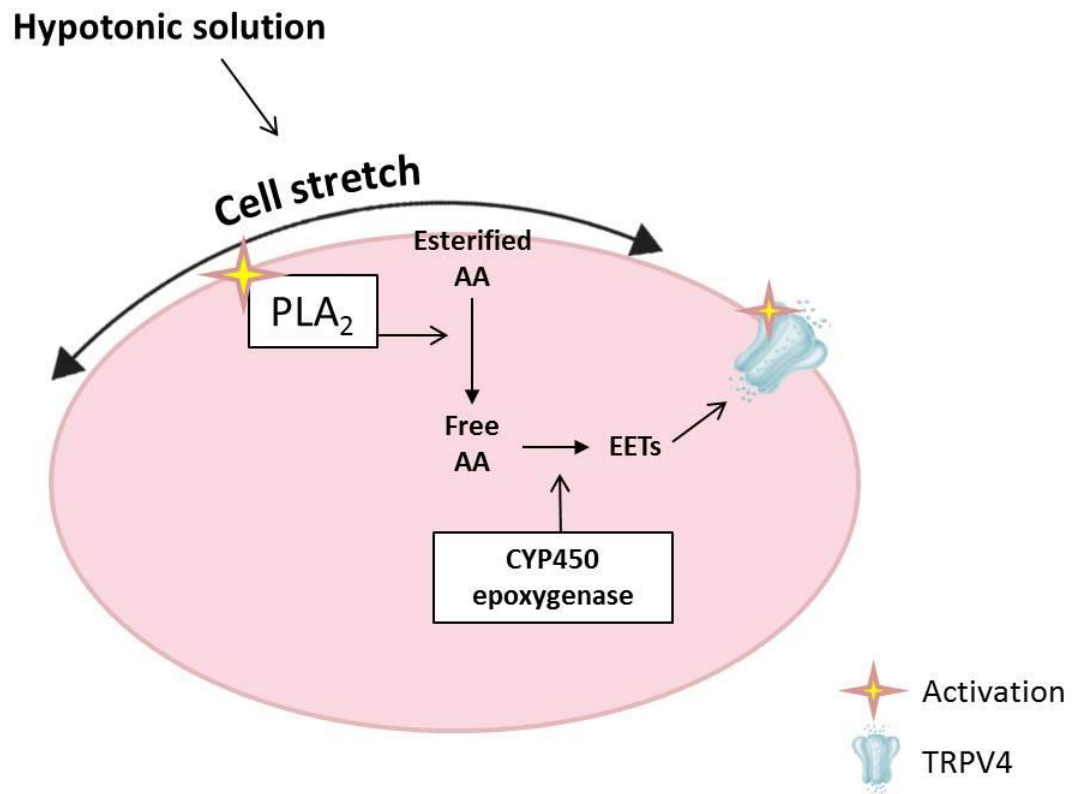


Figure 48. Putative signalling mechanisms for TRPV4 activation by changes in extracellular osmolarity. Decreases in extracellular osmolarity cause cell swelling and activation of phospholipase A₂ (PLA₂). PLA₂ catalyses the release of arachidonic acid (AA) from membrane phospholipids, which in turn is metabolised to epoxyeicosatrienoic acids (EETs) by cytochrome P450 (CYP450) epoxygenase. EETs may activate TRPV4 directly, or through the generation of further second messengers.

6.1.4.5 Phorbol ester derivatives

Watanabe and colleagues were the first to report activation of TRPV4 by phorbol ester derivatives (Watanabe et al., 2002a). The compound 4 α -phorbol 12,13 didecanoate (4 α PDD), in particular, was thought to activate TRPV4 exclusively, though recent studies have challenged its selectivity (Alexander et al., 2013). Responsiveness of TRPV4 to 4 α PDD is unaffected by PLA₂ inhibitors, though mutation of Tyr555 abolishes responses to both 4 α PDD and heat, suggesting a common pathway of activation for these two stimuli (Vriens et al., 2004). The divergent pathways of activation by cell swelling/arachidonic acid and by phorbol ester derivatives was subsequently confirmed in endothelial cells (Vriens et al., 2005). The response of TRPV4 to phorbol ester derivatives also appears to be temperature-dependent, however: higher temperatures potentiate responses, which are relatively diminished at RT (Gao et al., 2003).

6.1.4.6 Temperature gating

Like many other members of the TRPV subfamily, TRPV4 is gated by temperature. Whereas TRPV1 is activated by relatively hot temperatures ($\geq 42^{\circ}\text{C}$), the temperature threshold of TRPV4 is between 25 and 33 $^{\circ}\text{C}$ (Guler et al., 2002, Watanabe et al., 2002b); heat activates only a fraction of the number of channels responsive to 4 α PDD, suggesting less efficient channel recruitment (Watanabe et al., 2002b). Responses to warming exhibit a degree of desensitisation, though some spontaneous activity remains at 37 $^{\circ}\text{C}$ (Plant and Strotmann, 2007). TRPV4 responses to heat are observed in cell-attached but not cell-free patches (Watanabe et al., 2002b), indicative of an indirect activation that involves intracellular signalling components. The ankyrin repeat domains present in the N-terminus of the channel are thought to be essential for heat activation (Watanabe et al., 2002b).

Consistent with TRPV4 expression in sensory neurons, keratinocytes and the hypothalamus, TRPV4 KO mice exhibit altered physiological and behavioural responses to thermal stimuli (Todaka et al., 2004), particularly in the context of thermal hyperalgesia (Suzuki et al., 2003, Liedtke and Friedman, 2003, Todaka et al., 2004). Despite its expression in the hypothalamus, the centre of thermoregulation (Boulant, 1981), no difference in core temperature, or in the thermodynamic response to cold stress have been recorded in TRPV4 KO mice, however, indicating intact thermoregulatory function (Liedtke and Friedman, 2003).

6.1.4.7 Ca²⁺-dependent regulation of TRPV4 activity

Consistent with the cytotoxicity associated with excessive Ca²⁺ entry, negative-feedback regulatory mechanisms exist for most Ca²⁺-permeable channels. Ca²⁺ is involved in both the

activation phase and the decay phase of TRPV4 responses to hypotonicity, phorbol ester derivatives and heat (Plant and Strotmann, 2007). Removal of extracellular Ca^{2+} abolishes spontaneous TRPV4 activity and lowers intracellular $[\text{Ca}^{2+}]$. Replacement of extracellular Ca^{2+} with Ba^{2+} or Sr^{2+} – which also permeate the channel well – delay and blunt agonist-induced responses, highlighting the dependence of activation on Ca^{2+} (Strotmann et al., 2003).

Like TRPV1, TRPV4 contains a putative calmodulin binding site in its cytosolic C-terminus, through which Ca^{2+} -dependent regulation may be achieved (Strotmann et al., 2003). While mutations to this motif were found to alter Ca^{2+} -induced potentiation of channel activation by a variety of agonists, however, current inactivation was unaffected (Strotmann et al., 2003). Large currents through TRPV4 decay below baseline levels, even with persistent exposure to agonists, in a manner that is dependent upon Ca^{2+} : Watanabe and colleagues have shown that increasing extracellular $[\text{Ca}^{2+}]$ not only potentiates TRPV4 activation, but also accelerates and enhances current decay; furthermore, raising intracellular $[\text{Ca}^{2+}]$ with a patch pipette also reduces current amplitudes (Watanabe et al., 2003a). The mechanism by which Ca^{2+} inactivates TRPV4 remains unclear, however.

6.1.5 TRPV4 in vasoregulation

Consistent with its role as a Ca^{2+} channel, TRPV4 has been implicated in a variety of physiological processes, including nociception (Alessandri-Haber et al., 2003), bone development (Rock et al., 2008, Krakow et al., 2009) and osmoregulation (Liedtke and Friedman, 2003, Mizuno et al., 2003). This thesis will focus on its role in the cardiovascular system, where it is emerging as an important regulator of vascular function.

6.1.5.1 The role of TRPV4 in vascular endothelial cells

The first evidence of functional TRPV4 expression in the murine aortic endothelium was provided by Bernd Nilius' group (Watanabe et al., 2002b). Since then, its endothelial expression has been characterised throughout the vascular system, from large conductance vessels to the microvasculature (Filosa et al., 2013). Aortic expression of TRPV4 was confirmed by Vriens and colleagues using RT-PCR, Western blot analysis and functional Ca^{2+} imaging in isolated murine aortic endothelial cells (Vriens et al., 2005); similarly endothelial TRPV4 activity has been demonstrated in the rat carotid artery and arteria gracilis by *in situ* patch-clamping, single-cell RT-PCR and pressure myography (Kohler et al., 2006), and in the pulmonary artery and microvascular endothelium (Alvarez et al., 2006). More recently, a systemic study of rat tissues by Willette and colleagues has revealed a ubiquitous pattern of TRPV4-positive staining

in endothelial and epithelial tissues (Willette et al., 2008). Data outlined in **Chapter 5** of this thesis further support a functional role for TRPV4 in the vascular endothelium.

The function of TRPV4 in endothelial tissue has been the subject of intense investigation in recent years, and the generation of TRPV4 KO mice (Liedtke and Friedman, 2003) has helped to elucidate the channel's role in vascular regulation. Many studies have implicated TRPV4 in flow- and agonist-induced vasodilation in a variety of rodent vascular beds (Kohler et al., 2006, Hartmannsgruber et al., 2007, Loot et al., 2008, Zhang et al., 2009, Mendoza et al., 2010, Adapala et al., 2011, Zhang and Gutterman, 2011)

Zhang and colleagues were among the first to demonstrate a role for TRPV4 in acetylcholine (ACh)-mediated vasorelaxation. ACh-induced nitric oxide (NO) production was found to be significantly reduced in carotid artery endothelial cells from TRPV4 KO mice; in TRPV4 KO small mesenteric arteries, the NO-mediated component of ACh-induced vasodilatation was attenuated and the endothelium derived hyperpolarising factor (EDHF)-mediated component abolished (Zhang et al., 2009). The haemodynamic depressor response to ACh was similarly attenuated in TRPV4 KO mice (Zhang et al., 2009). These data suggest that TRPV4 is involved in ACh-mediated vasorelaxation, in a manner that is consistent with the relative contribution of endothelium-derived relaxing factors in different vascular beds: in larger conduit vessels, NO and prostanoids are the principal mediators, whereas in smaller resistance beds, EDHF predominates (Hwa et al., 1994). The results of Zhang and colleagues are furthermore consistent with a previous report showing that in larger vessels, inhibition of endothelial nitric oxide synthase (eNOS) almost completely abolished 4αPDD-induced vasodilatation, whereas in smaller vessels, selective blockade of small- and intermediate-conductance Ca^{2+} -sensitive SK_{Ca} and IK_{Ca} channels inhibited TRPV4-mediated vasodilatation (Kohler and Hoyer, 2007). Numerous studies have indicated that TRPV4 activation may represent the hyperpolarising mechanism by which ACh induces endothelium-dependent vasodilatation.

In addition to vasoactive responses to agonist stimulation, TRPV4 has also been implicated in the vasodilatory response to fluid shear stress (Hartmannsgruber et al., 2007, Mendoza et al., 2010, Loot et al., 2008). Mendoza and colleagues showed that TRPV4-mediated Ca^{2+} influx is essential for flow-mediated dilatation of human coronary arteries and involves CYP450 epoxygenase activity and PLA_2 (Mendoza et al., 2010); this has also been demonstrated in murine carotid arteries (Loot et al., 2008, Hartmannsgruber et al., 2007). Pre-constricted murine carotid arteries exhibit a predominantly NO-independent and entirely prostacyclin-independent vasodilatory response to increases in fluid shear stress. This response is abolished

in conditions of high extracellular $[K^+]$ and following inhibition of CYP450 epoxygenase activity (Loot et al., 2008).

In 2012, Sonkusare and colleagues used a very elegant approach to investigate the role of TRPV4 in the endothelium of small resistance arteries. Using transgenic mice expressing a Ca^{2+} biosensor exclusively in the endothelium, the authors recorded basal endothelial Ca^{2+} pulses in isolated mesenteric arteries, that were dramatically increased in frequency by the administration of 10 nM GSK1016790A, a selective TRPV4 agonist (Sonkusare et al., 2012). In contrast to Ca^{2+} pulsars, which tend to be localised to holes in the internal elastic lamina (representing sites of endothelial projections into the smooth muscle), the Ca^{2+} sparklets described by Sonkusare and colleagues were evenly distributed between myoendothelial holes and the ends of cells. Rapid removal of extracellular Ca^{2+} abolished the responses to GSK1016790A.

Stimulation with 10 nM GSK1016790A was found to activate approximately three sparklets per cell, though this was sufficient to hyperpolarise the underlying smooth muscle and cause maximal relaxation of the pressurised arteries. This dilatory response was unaffected by inhibitors of eNOS and cyclo-oxygenase, though charybdotoxin – an inhibitor of intermediate-conductance IK_{Ca} channels – abolished dilatation in response to 3 nM GSK1016790A, and greatly reduced the dilatory response to 10 nM GSK1016790A. Perforated patch clamping revealed an outward current in response to 10 nM GSK1016790A that was substantially reduced by charybdotoxin and further reduced by subsequent addition of apamin – a blocker of small-conductance SK_{Ca} channels. In fact, 10 nM GSK1016790A was found to increase the density of IK^+ and SK^+ currents by a similar magnitude to dialysis with 3 μM Ca^{2+} . Together, these results demonstrate a specific link between TRPV4 activation and endothelium-dependent hyperpolarisation of vascular smooth muscle: a hyperpolarising current generated in endothelial cells may spread to the underlying smooth muscle via myoendothelial gap junctions, leading to vascular relaxation.

Zheng and colleagues provided the first evidence of TRPV4-dependent endothelial regulation in human vessels. The authors demonstrated that arachidonic acid-induced dilatation of isolated human coronary arterioles depends on endothelial TRPV4-mediated Ca^{2+} entry and endothelial hyperpolarisation via activation of IK_{Ca} and SK_{Ca} channels (Zheng et al., 2013). They demonstrated in isolated human coronary artery endothelial cells that arachidonic acid causes TRPV4-dependent Ca^{2+} entry that is markedly decreased following inhibition of protein kinase A (PKA). The PKA activator forskolin had no effect on Ca^{2+} entry, suggesting that protein

phosphorylation facilitates activation of TRPV4 by stimuli such as arachidonic acid, without directly opening the channel.

6.1.5.2 The role of TRPV4 in vascular smooth muscle cells

In addition to mediating endothelium-dependent smooth muscle hyperpolarisation, TRPV4 may contribute to smooth muscle hyperpolarisation directly. TRPV4 expression has been localised to the smooth muscle cells of rat cerebral arteries (Marrelli et al., 2007, Earley et al., 2005), human and rat lung extra-alveolar vessels (Alvarez et al., 2006), endothelium-denuded rat intralobar pulmonary arteries (Martin et al., 2012, Yang et al., 2012), rat and mouse mesenteric arteries (Gao and Wang, 2010, Earley et al., 2009), and rat and mouse aortae (Watanabe et al., 2008). Despite representing a Ca^{2+} entry pathway, activation of TRPV4 in smooth muscle cells is associated with smooth muscle hyperpolarisation and vascular relaxation.

Under high-speed laser-scanning confocal microscopy, both 4αPDD and 11,12-EET have been shown to elicit Ca^{2+} sparks via sarcoplasmic ryanodine receptors in the smooth muscle cells of isolated rat cerebral arteries (Earley et al., 2005). These sparks were found to activate sarcolemmal large-conductance BK_{Ca} channels, leading to smooth muscle hyperpolarisation and relaxation. These responses were abolished following anti-sense knockdown of TRPV4 expression (Earley et al., 2005), suggesting that TRPV4 may form a Ca^{2+} signalling complex with ryanodine receptors and BK_{Ca} channels to mediate smooth muscle relaxation; endogenous activation of this complex may involve endothelium-derived relaxing factors, such as EETs.

Further studies of TRPV4 function have been conducted in murine small mesenteric arteries: 11,12-EET-induced ion currents and vasorelaxation were abolished in arteries isolated from TRPV4 KO mice (Earley et al., 2009). Endothelial denudation reduced 11,12-EET-induced smooth muscle hyperpolarisation and relaxation by approximately 50%, implicating smooth muscle-derived TRPV4 in the remaining response. Correspondingly, the authors found that inhibition of endothelial IK_{Ca} and SK_{Ca} channels, or smooth muscle BK_{Ca} channels similarly attenuated 11,12-EET-induced responses to an equivalent degree.

EETs can be produced by endothelial cells in response to numerous physiological stimuli, including bradykinin, ACh, pulsatile or shear stress (Filosa et al., 2013). Thus EET-induced activation of endothelial and smooth muscle TRPV4 channels, via coupling to K_{Ca} channels, may play a major role in the control of vascular tone under various physiological conditions.

Consistent with this view, TRPV4 KO mice exhibit exaggerated hypertension in response to chronic eNOS inhibition, relative to WT controls (Earley et al., 2009).

Until now, a functional role for TRPV4 in vascular smooth muscle has only been shown definitively in rat cerebral arteries (Earley et al., 2005) and murine mesenteric arteries (Earley et al., 2009). It seems likely, however, that this mechanism is conserved throughout a variety of vascular beds: indirect stimulation of hyperpolarising smooth muscle BK_{Ca} channels by EETs has been demonstrated in numerous artery types (Campbell and Fleming, 2010), supporting a widespread role for the EET-TRPV4-BK_{Ca} axis in smooth muscle regulation throughout the vascular system.

6.1.5.3 Other mechanisms of vasoregulation

In addition to its expression in vascular tissue, TRPV4 is also thought to be present in perivascular nerves, where it co-localises with vasoactive peptides CGRP and SP (Grant et al., 2007, Koltzenburg, 2004). Both sensory denervation and CGRP antagonism have been shown to attenuate the hypotensive response to 4αPDD (Gao and Wang, 2010), implicating neuronal TRPV4 expression in haemodynamic regulation. Other studies have suggested that TRPV4 expressed in DRG neurons may initiate a sympathetic pressor response after sensing changes in osmolarity in the portal vascular bed (McHugh et al., 2010).

6.1.6 A potential role for TRPV4 in sepsis

Systemic activation of TRPV4 is known to cause profound hypotension and circulatory collapse (Pankey et al., 2014, Willette et al., 2008) – key features of septic shock. In addition to its potential involvement in the regulation of vascular tone, TRPV4 has also been implicated in the endothelial retraction and barrier failure (Willette et al., 2008); it is known to contribute to pulmonary oedema in response to raised pulmonary venous pressure (Alvarez et al., 2006, Wu et al., 2009, Hamanaka et al., 2010) – as encountered in sepsis (Parker and Brigham, 1987, D'Orio et al., 1986). An elevation in lung microvascular pressure has been shown to elicit Ca²⁺ influx via TRPV4; this, in addition to activating myosin light chain kinase (MLCK) to initiate endothelial retraction (increasing endothelial permeability and oedema formation), also induces NO formation which acts as a negative feedback mediator to switch off TRPV4 activity through a cGMP-dependent mechanism (Yin et al., 2008). In cases of low NO bioavailability (as encountered in sepsis, where elevated ROS production can lead to peroxynitrite formation), this negative-feedback loop may become compromised, leading to elevated pulmonary oedema formation.

Low NO bioavailability may also alter vasoactive responses to TRPV4 activation. In the presence of L-NAME, pulmonary arteries pre-constricted with U-46619 have been shown to respond to TRPV4 activation in a biphasic manner, with a potent isradipine-sensitive vasoconstrictor component (Pankey et al., 2014). Thus, in sepsis, where excessive ROS production and insufficient L-arginine production can lead to low NO bioavailability (Luiking et al., 2009), TRPV4-mediated Ca^{2+} entry may elicit vasoconstriction, exacerbating impaired perfusion in microvascular beds.

Numerous endogenous agonists and regulators of TRPV4, including anandamide (Varga et al., 1998), arachidonic acid and its metabolites (Bruegel et al., 2012), and PKA (Yang et al., 1997), have been shown to be upregulated in sepsis. It is conceivable therefore, that inflammation-induced upregulation of endogenous factors could contribute to sepsis-associated circulatory failure via inappropriate TRPV4 activation. Alternatively, because TRPV4 is thought to be constitutively active at body temperature (Strotmann et al., 2000), it is possible that inflammatory mediators may sensitise the channel and increase tonic activity.

6.1.7 Hypotheses

Given that TRPV4 may be sensitised in inflammation (as demonstrated in **Chapter 5**) and that excessive TRPV4 activation is associated with profound hypotension, endothelial failure and circulatory collapse (Willette et al., 2008), it was hypothesised that deletion or blockade of TRPV4 will be protective against cardiovascular dysfunction in sepsis.

6.1.8 Aims

The aims of this study were as follows:

1. To compare haemodynamic performance using radiotelemetry in TRPV4 KO and WT mice, treated with either vehicle or TRPV4 antagonist, both at baseline and during endotoxaemia
2. To use the novel model of microcirculatory assessment described in **Chapter 3** to determine the impact of TRPV4 deletion or antagonism on microcirculatory blood flow under basal and endotoxaemic conditions
3. To relate any changes in haemodynamic performance and blood flow to other measurements of disease severity, including blood markers of acidosis and organ dysfunction, and other macroscopic clinical parameters
4. To determine the impact of topical pharmacological TRPV4 activation on mesenteric blood flow in healthy and endotoxaemic WT mice

6.2 Methods

6.2.1 Materials

A list of materials used in this study, and details of their origin, are provided in **Table 12**.

Material	Manufacturer	Catalogue No
GSK1016790A	Sigma-Aldrich	G0798
HC-067047	Tocris	4100
Lipopolysaccharide from <i>Salmonella typhimurium</i>	Sigma-Aldrich	L7261

Table 12. Materials used in study.

6.2.2 Animals

Male wildtype (WT) and TRPV4 knockout (KO) mice were bred in house from heterozygous crosses of breeding pairs kindly donated by Dr Andrew Grant (Wolfson Centre for Age-Related Diseases, King's College London), as described in section **2.1**. Mice were used between 10 and 14 weeks of age (approx. 25–30g).

6.2.3 Haemodynamic recording in the transition from health to disease

Radiotelemetry catheters were introduced into the carotid artery of 10-week old mice, as described in section **2.4**. Animals were allowed to recover over 10 days before baseline haemodynamic parameters were recorded over a 48-h weekend period. Food and water intake was measured continuously, and mice were closely monitored for adverse signs. The following weekend, HC-067047 (10 mg/kg in 500 μ L, i.p.) or vehicle (10% DMSO in 500 μ L, i.p.) was administered to WT mice by Dr Anna Starr or Dr Manasi Nandi for blinding purposes, based on doses characterised previously (Everaerts et al., 2010). TRPV4 KO mice were left untreated. Haemodynamic parameters were then recorded over a second 48-h weekend period. On the following Monday, WT mice were re-injected with either HC-067047 or vehicle (consistent with the first treatment) and lipopolysaccharide (LPS; 12.5mg/kg) from *Salmonella typhimurium* (Sigma, L7261) was administered intravenously to all mice, as described in section **2.3.3.1**. Haemodynamic parameters were recorded for 24 h, and mice were then terminally anaesthetised for blood flow assessment, as described in **Chapter 3**.

6.2.4 *In situ* monitoring of mesenteric blood flow

Mesenteric blood flow was measured in naïve and septic mice 24 h post-LPS, as described in **Chapter 3**. After 5 min baseline recording, the mesenteric bed was gently sprayed (2 pump compressions from a distance of approximately 10 cm) with 1 μ M GSK1016790A dissolved in saline containing 1% DMSO, and pre-warmed to 37°C. Responsiveness to GSK1016790A was measured over a further 5 min; a venous blood sample was subsequently drawn from the inferior vena cava, and animals were terminated by cervical dislocation. Blood biochemistry was assessed immediately from 100 μ l venous blood using a hand-held iSTAT® point-of-care analyser (Abbott Laboratories, IL, USA), with CG8+ cartridges (Abbott Laboratories). Results were analysed in moorFLPI Review software (V4.0) and GraphPad Prism, as described in section **3.2.7.1**. Results are expressed as mean area under the curve (AUC) over time \pm SEM.

6.2.5 Assessment of organ oedema

Oedema formation was measured by comparing wet and dry weights of lungs, hearts, upper liver lobes, right kidneys, 1-cm sections of small intestine and whole spleens. Tissues were excised immediately after blood flow recording and wet weights were recorded. Samples were then placed in an oven at 50°C until a constant dry weight was reached (approximately 3 days). The ratio of wet weight:dry weight was used as an indication of oedema formation.

A timeline summarising the sequence of experimental procedures is provided in **Figure 49**.

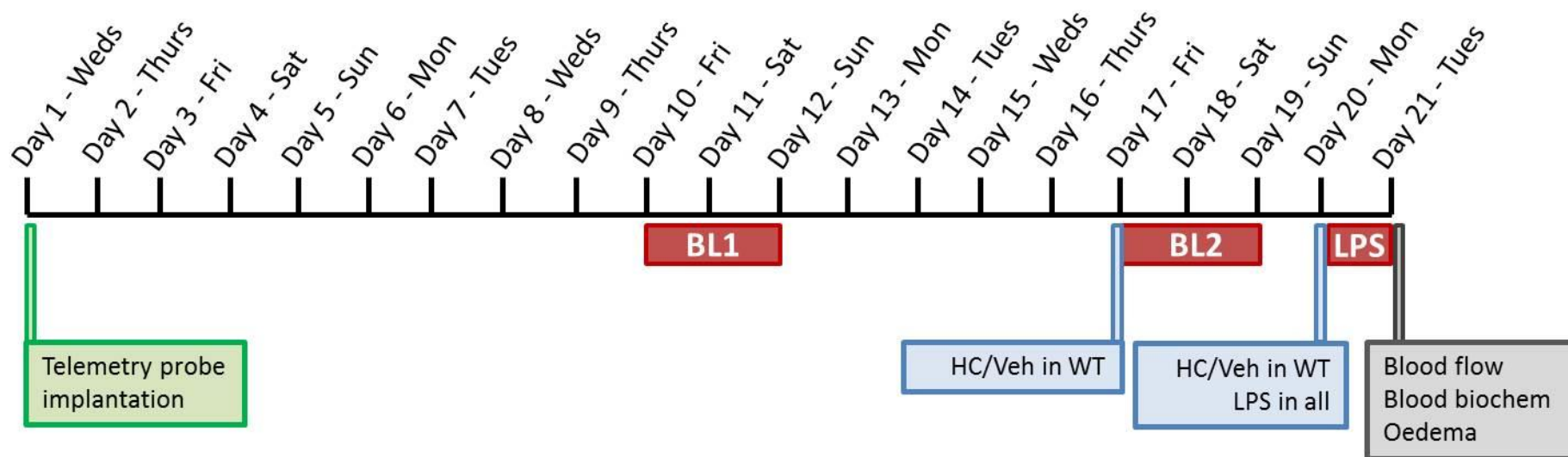


Figure 49. Timeline of experimental procedures. Mice were implanted with telemetry probes on Day 1, and were left to recover for 10 days. Basal haemodynamic data were then recorded over a 48-h weekend period (BL1). The following weekend, WT mice were injected with either vehicle (10% DMSO in saline) or TRPV4 antagonist HC-067047 (10 mg/kg, i.p.) and a second 48-h baseline recording (BL2) was taken for all mice. TRPV4 KO mice were left untreated. Injections in WT mice were performed in a randomised fashion by Dr Anna Starr or Dr Manasi Nandi to ensure investigator blinding. Following the second weekend baseline recording, WT mice received a second injection of either HC-067047 or vehicle (consistent with initial treatment) and LPS (12.5 mg/kg, i.v.) was administered to all mice. Haemodynamic data were recorded over 24-h of sepsis progression. Mice were then terminally anaesthetised for blood flow recording and *ex vivo* analysis. A separate cohort of naïve mice were included in blood flow and *ex vivo* studies.

6.3 Results

6.3.1 TRPV4 heterozygous mice did not breed according to expected Mendelian ratios

C57/BL6 mice heterozygous for a non-functional TRPV4 receptor gene (TRPV4 KO) were re-derived from frozen embryos generously donated by Dr Andy Grant, Wolfson Centre for Age-Related Disease, and were originally derived from a colony generated by Liedtke & Friedman at the Rockefeller Institute in New York (Liedtke and Friedman, 2003). While mice in Dr Grant's colony were found to breed normally, heterozygous crosses set up in our animal unit did not yield expected Mendelian ratios: only 5 male TRPV4 KOs were born following multiple rounds of breeding by 8 heterozygous pairs (total >300 pups). Typical genotyping results are shown in **Fig. 50**.

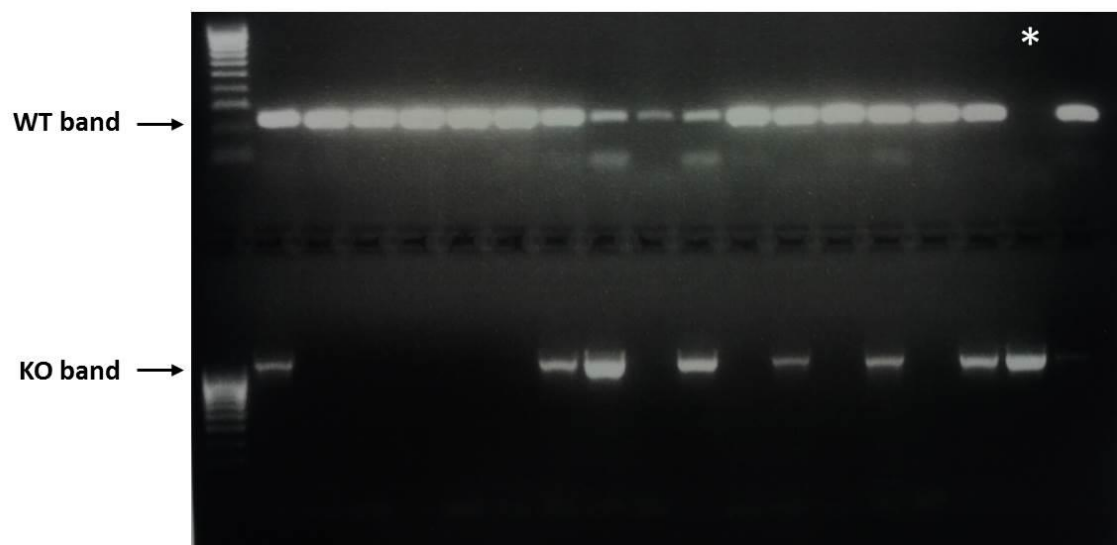


Figure 50. Typical genotyping results from heterozygous cross. Crosses of TRPV4 heterozygotes generated predominantly WT (single upper band) and heterozygous (upper and lower bands) pups. Only one KO (marker by the asterisk) was born in this litter.

6.3.2 Loss of TRPV4 activity is associated with modest alterations in water but not food intake

TRPV4 WT and KO mice were singly-housed from the time of radiotelemetry implantation throughout the duration of the study. Food and water intake were monitored throughout. Recovery from telemetry surgery (measured as recovery in body weight and food and water intake) was equivalent across all animals. Following recovery, TRPV4 KO mice were found to consume marginally less water than WT counterparts, though this difference was not statistically significant (**Fig. 51a**). Systemic administration of both HC-067047 and vehicle reduced water intake, though the magnitude of change was significantly greater in antagonist-treated animals (**Fig. 51b**). Water intake was reduced in all animals over the 24 h following the induction of sepsis (**Fig. 51c**). No difference in food intake was observed between different genotypes (**Fig. 51d**). Systemic administration of both vehicle and HC-067047 slightly reduced food intake, though to a similar degree across treatment groups (**Fig. 51e**). LPS markedly reduced food intake to a similar degree in all groups (**Fig. 51f**).

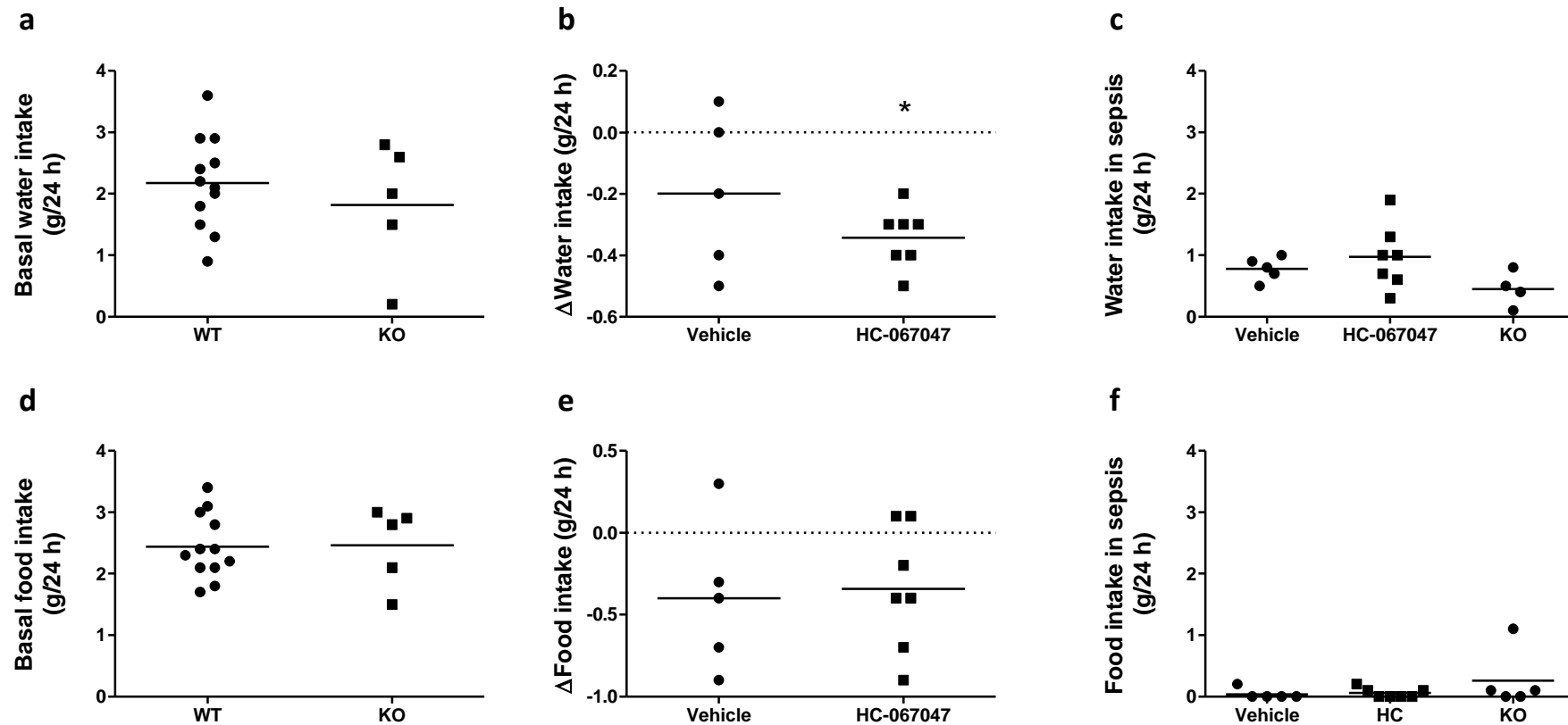


Figure 51. Water and food intake in TRPV4 WT and KO mice before and after induction of sepsis. (a) Mean basal water intake in TRPV4 WT and KO mice over approximately 1 week (not including 7-day recovery period from telemetry surgery). (b) Change in water intake over 24-h period following i.p. administration of either vehicle (10% DMSO) or HC-067047. (c) Water intake over 24-h period following administration of LPS (12.5 mg/kg i.v.). (d) Mean basal food intake in TRPV4 WT and KO mice over approximately 1 week (not including 7-day recovery period from telemetry surgery). (e) Change in food intake over 24-h period following i.p. administration of either vehicle (10% DMSO) or HC-067047. (f) Food intake over 24-h period following administration of LPS (12.5 mg/kg i.v.). Data for individual animals are represented as individual symbols, with group mean denoted by horizontal line. * $p < 0.05$, relative to vehicle-treated controls, 1-way ANOVA + Bonferroni post-hoc test.

6.3.3 Loss of TRPV4 activity is associated with modest haemodynamic alterations

Male TRPV4 WT and KO littermates were implanted with radiotelemetry devices for remote haemodynamic monitoring. Two WT mice were excluded from the study due to loss of transmitter signal. Under basal conditions, all animals exhibited diurnal variation in blood pressure, heart rate and ambulatory activity, within the expected physiological range for mice (Curtis et al., 2007) (**Fig. 52**). No significant difference in basal haemodynamic parameters was observed between genotypes, although TRPV4 KO mice exhibited a trend towards slightly lower blood pressure (both daytime and night-time) and daytime ambulatory activity than WT counterparts [mean MAP over 24 h: 93.71 ± 2.83 mmHg vs. 99.89 ± 1.87 mmHg, respectively, ns; mean daytime activity over 12 h: 3.25 ± 0.43 counts/min vs. 4.31 ± 0.33 counts/min, respectively, ns ($n = 5-10$)] (**Fig. 52a,c,d,f**).

Systemic administration of the TRPV4 antagonist HC-067047 caused an increase in mean daytime blood pressure of approximately 5 mmHg, though this was reversed after 12 h, leading to small but consistent decrease in night-time pressure (**Table 13**). Systemic administration of vehicle (10% DMSO in saline) also caused an increase in blood pressure, however, though this was more transient (**Fig. 52**). Administration of neither HC-067047 nor vehicle significantly altered any other haemodynamic parameters under basal conditions, though heart rate was transiently increased in both groups, and subsequently declined during night-time hours, relative to time-matched pre-treatment values (**Table 13**). In both cases (daytime tachycardia and night-time bradycardia) the magnitude of the change was slightly greater in HC-067047-treated mice than in vehicle-treated controls, though this was not statistically significant. Mice treated with HC-067047 did exhibit significantly greater daytime ambulatory activity, relative to time-matched pre-treatment values (**Table 13**); this change was not observed in vehicle-treated animals, and was no longer evident during night-time hours.

Since TRPV4 KO mice were not treated with pharmacological stimuli, baseline parameters were simply re-recorded in the same mice. Values were relatively consistent across separate baseline recording periods (**Table 13**), confirming the robustness of the monitoring system.

Systemic administration of LPS caused a significant decline in blood pressure in all animals (**Fig. 52a, c, d & Table 13**), that began to stabilise after approximately 12 h. No significant difference between genotypes was observed, though both TRPV4 KOs and antagonist-treated mice exhibited a trend towards more severe hypotension, particularly in terms of systolic pressure (**Fig. 52a & c and Table 13**). Pulse pressure also appeared more severely compromised in TRPV4 KO and HC-067047-treated mice, relative to vehicle-treated controls, a potential sign of

left ventricular dysfunction and low stroke volume and/or excessive aortic compliance (**Fig. 52e** and **Table 13**).

An immediate increase in heart rate was observed in both HC-067047- and vehicle-treated WT animals following the induction of sepsis; this tachycardia was sustained during daylight hours, consistent with a baroreceptor reflex to low blood pressure. TRPV4 KO mice exhibited a very transient increase in heart rate immediately after LPS administration, but became markedly bradycardic thereafter, indicating failure of the baroreceptor reflex (**Fig. 52b** and **Table 13**). All mice exhibited marked bradycardia during night-time hours, relative to time-matched pre-LPS values, though the magnitude of the decrease was greatest in TRPV4 KO mice (**Table 13**). Ambulatory activity was almost entirely abolished in all mice following the induction of sepsis (**Fig. 52f**).

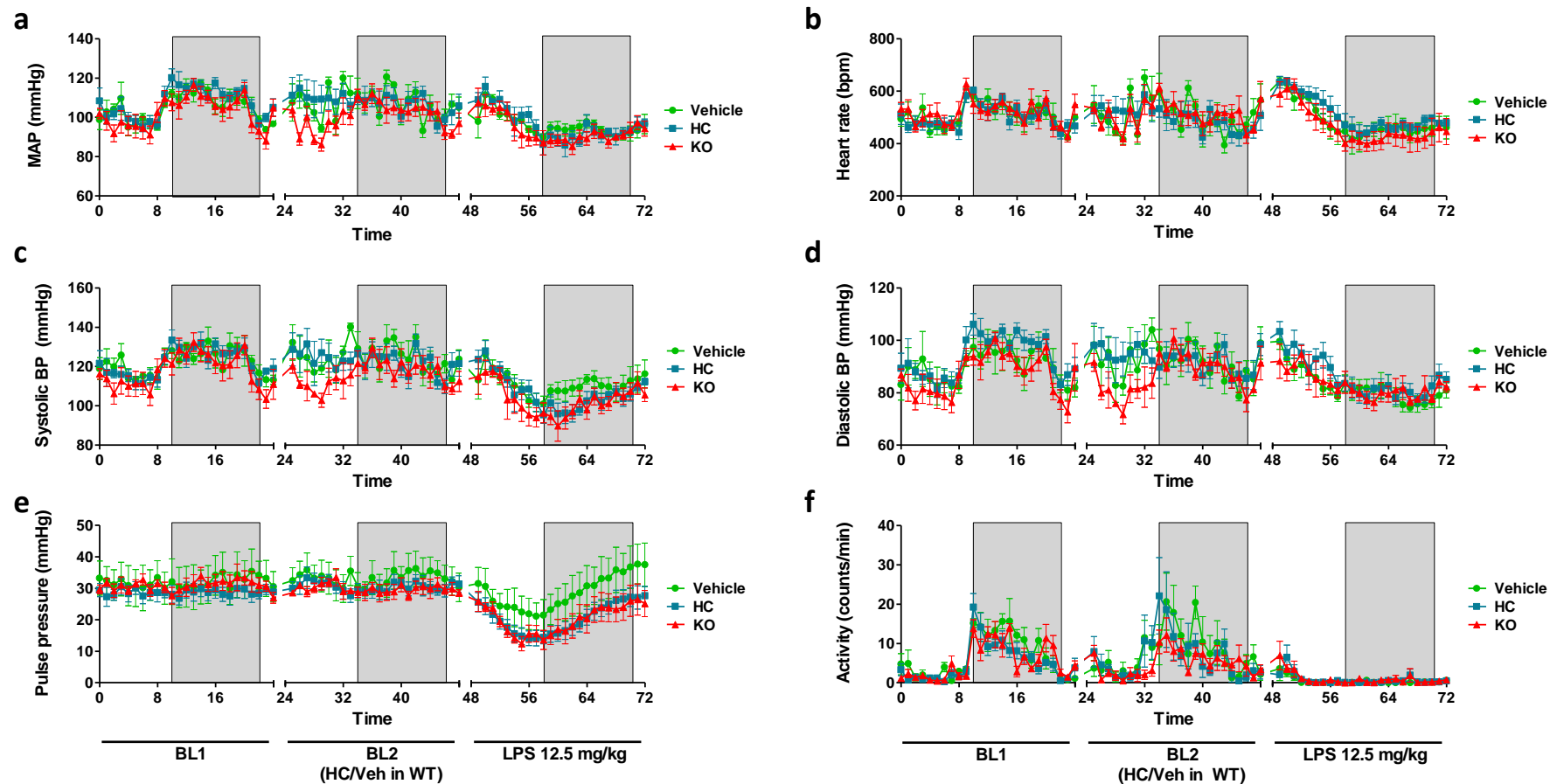


Figure 52. Haemodynamic monitoring in TRPV4 WT and KO mice under basal and septic conditions. (a) Mean arterial pressure, (b) heart rate, (c) systolic pressure, (d) diastolic pressure, (e) pulse pressure and (f) locomotor activity at baseline (BL1), following systemic treatment (WT mice only) with HC-067047 (HC) or vehicle (10% DMSO) (BL2) and after induction of sepsis (at time 48h: LPS, 12.5mg/kg, i.v.). Boxed regions denote periods of darkness. Data were acquired by radiotelemetry in conscious, ambulatory male C57BL/6 mice, and are presented as mean \pm SEM, $n = 4-6$.

Mean arterial pressure, mean diff (95% CI), mm Hg						
	Day			Night		
Comparison	WT + Vehicle (n = 4)	WT + HC-067047 (n = 6)	TRPV4 KO (n = 5)	WT + Vehicle (n = 4)	WT + HC-067047 (n = 6)	TRPV4 KO (n = 5)
BL1 – BL2	+4.835 (-10.99 to +20.66)	+5.297 (-7.62 to +18.21)	-0.406 (-14.56 to +13.74)	-1.532 (-13.82 to +10.75)	-4.956 (-14.99 to +5.08)	-2.327 (-13.32 to +8.66)
BL1 – LPS	-2.838 (-18.66 to +12.98)	-2.124 (-15.04 to +10.79)	-4.326 (-18.48 to +9.825)	-14.480** (-29.77 to +2.19)	-19.020*** (-29.05 to -8.99)	-15.410*** (-26.4 to -4.418)
BL2 – LPS	-7.674 (-23.49 to +8.147)	-7.421 (-20.34 to +5.50)	-3.920 (-18.07 to +10.23)	-12.95* (-25.23 to +0.66)	-14.060 (-24.09 to -4.03)	-13.080** (-24.07 to -2.09)
Systolic pressure, mean diff (95% CI), mm Hg						
	Day			Night		
Comparison	WT + Vehicle (n = 4)	WT + HC-067047 (n = 6)	TRPV4 KO (n = 5)	WT + Vehicle (n = 4)	WT + HC-067047 (n = 6)	TRPV4 KO (n = 5)
BL1 – BL2	+4.523 (-14.82 to +23.87)	+5.443 (-10.35 to +21.24)	-1.898 (-19.20 to +15.41)	-0.935 (-19.33 to +17.46)	-3.393 (-18.41 to +11.62)	-2.923 (-19.37 to +13.53)
BL1 – LPS	-7.558 (-26.90 to +11.79)	-6.681 (-22.48 to +9.12)	-9.699 (-27.00 to +7.61)	-14.64 (-33.03 to +3.75)	-22.590*** (-37.61 to -7.58)	-19.870** (-36.32 to -3.42)
BL2 – LPS	-12.08 (-31.43 to +7.27)	-12.12 (-27.92 to +3.67)	-7.801 (-25.10 to +9.50)	-13.70 (-32.10 to +4.69)	-19.20** (-34.22 to -4.19)	-16.94* (-33.39 to -0.49)

Diastolic pressure, mean diff (95% CI), mm Hg						
	Day			Night		
Comparison	WT + Vehicle (n = 4)	WT + HC-067047 (n = 6)	TRPV4 KO (n = 5)	WT + Vehicle (n = 4)	WT + HC-067047 (n = 6)	TRPV4 KO (n = 5)
BL1 – BL2	+4.175 (-12.49 to +20.84)	+3.614 (-10.00 to +17.22)	-0.3836 (-15.29 to +14.53)	-1.603 (-13.95 to +10.75)	-6.103 (-16.19 to +3.98)	-0.263 (-11.31 to +10.78)
BL1 – LPS	-0.366 (-17.04 to +16.30)	+1.581 (-12.03 to +15.19)	+2.953 (-11.96 to +17.86)	-13.410* (-25.76 to -1.06)	-15.940*** (-26.03 to -5.86)	-9.740 (-20.79 to +1.307)
BL2 – LPS	-4.541 (-21.21 to +12.13)	-2.033 (-15.64 to +11.58)	+3.337 (-11.57 to +18.25)	-11.810 (-24.16 to +0.54)	-9.841 (-19.93 to +0.24)	-9.476 (-20.52 to +1.57)
Heart rate, mean diff (95% CI), bpm						
	Day			Night		
Comparison	WT + Vehicle (n = 4)	WT + HC-067047 (n = 6)	TRPV4 KO (n = 5)	WT + Vehicle (n = 4)	WT + HC-067047 (n = 6)	TRPV4 KO (n = 5)
BL1 – BL2	+18.45 (-80.45 to +117.40)	+22.51 (-58.25 to +103.30)	-0.96 (-89.43 to +87.50)	-19.20 (-126.00 to +87.55)	-34.53 (-121.70 to +52.63)	-10.50 (-106 to +84.98)
BL1 – LPS	+23.19 (-75.75 to +122.10)	+56.30 (-24.46 to +137.10)	-2.04 (-90.50 to +86.42)	-57.22 (-164.00 to +49.54)	-42.61 (-129.80 to +44.55)	-86.79 (-182.30 to +8.69)
BL2 – LPS	+4.74 (-94.17 to 103.60)	+33.79 (-46.97 to +114.50)	-1.08 (-89.54 to +87.39)	-38.01 (-144.80 to +68.74)	-8.076 (-95.24 to +79.09)	-76.29 (-171.80 to +19.20)

Pulse pressure, mean diff (95% CI), mm Hg						
Day			Night			
Comparison	WT + Vehicle (n = 4)	WT + HC-067047 (n = 6)	TRPV4 KO (n = 5)	WT + Vehicle (n = 4)	WT + HC-067047 (n = 6)	TRPV4 KO (n = 5)
BL1 – BL2	+1.049 (-13.99 to +16.08)	+1.941 (-10.33 to +14.22)	-0.475 (-13.92 to +12.97)	+1.003 (-17.85 to +19.86)	+1.149 (-14.24 to +16.54)	-2.314 (-19.18 to +14.55)
BL1 – LPS	-6.405 (-21.44 to +8.63)	-9.437 (-21.71 to +2.84)	-11.62 (-25.06 to +1.83)	-0.939 (-19.79 to +17.91)	-6.659 (-22.05 to +8.74)	-9.965 (-26.83 to +6.90)
BL2 – LPS	-7.454 (-22.49 to +7.58)	-11.38 (-23.65 to +0.90)	-11.14 (-24.59 to +2.31)	-1.943 (-20.80 to +16.91)	-7.808 (-23.20 to +7.59)	-7.651 (-24.51 to +9.21)
Activity, mean diff (95% CI), counts/min						
Day			Night			
Comparison	WT + Vehicle (n = 4)	WT + HC-067047 (n = 6)	TRPV4 KO (n = 5)	WT + Vehicle (n = 4)	WT + HC-067047 (n = 6)	TRPV4 KO (n = 5)
BL1 – BL2	+1.548 (-1.97 to +5.07)	+3.260* (+0.39 to +6.14)	+0.880 (-2.27 to +4.03)	-0.114 (-4.20 to -3.97)	-0.360 (-3.69 to +2.97)	-2.169 (-3.30 to +3.69)
BL1 – LPS	-3.764 (-7.29 to +0.24)	-2.762 (-5.64 to +0.11)	-1.568 (-4.72 to +1.58)	-8.932*** (-13.02 to -4.85)	-5.788*** (-9.12 to -2.45)	-7.105*** (-10.76 to -3.45)
BL2 – LPS	-5.311*** (-8.83 to +1.79)	-6.023*** (-8.90 to -3.15)	-2.448 (-5.60 to +0.70)	-8.818*** (-12.90 to -4.74)	-5.428*** (-8.76 to -2.09)	-4.936** (-8.59 to -1.28)

Table 13. Statistical comparison of haemodynamic parameters in TRPV4 WT and KO mice under basal and septic conditions. Data are presented as mean difference (95% CI), and are divided into daytime and night-time phases. BL1 represents mean baseline values in untreated mice over 48-h weekend recording. BL2 represents mean baseline values following i.p. treatment (WT mice only) with vehicle (10% DMSO) or HC-067047; BL2 in KO mice represents a separate untreated 48-h baseline recording. LPS represents mean values following systemic treatment with LPS (12.5 mg/kg, i.v.) over 24-h recording period. *p<0.05, **p<0.01, ***p<0.001, 1-way ANOVA + Bonferroni post-hoc test.

6.3.4 Loss of TRPV4 activity does not significantly alter mesenteric blood flow

All mice telemetered for haemodynamic recording were subjected to mesenteric blood flow analysis. A group of naïve animals were included for comparison, and additional vehicle- and HC-067047-treated WT mice were used to increase statistical power. Owing to the low yield of TRPV4 KO mice, an additional 4 LPS-treated age-matched mice were included from the colony of Dr Andy Grant at the Wolfson Centre for Age-Related Diseases, King's College London; 6 naïve KO mice were also generously supplied by Dr Grant, though these were not age-matched. Blood flow recording in the 10 additional KO mice was performed on-site at the Wolfson Centre, as transfer to our unit was not possible.

Blood flow was significantly reduced in all groups 24 h after the induction of sepsis (**Fig. 53a–c**). No significant differences were observed between genotypes or treatment groups, though septic mice pre-treated with HC-067047 exhibited a slight trend towards improved flow. In order to determine whether blockade of TRPV4 may play a role earlier in sepsis pathogenesis, mesenteric flow was recorded in a separate cohort of WT mice treated for 6 h with HC-067047 and LPS. At this time-point, blood flow in HC-067047-treated mice was slightly decreased relative to vehicle-treated controls, though these changes were not statistically significant (**Fig. 53e–f**).

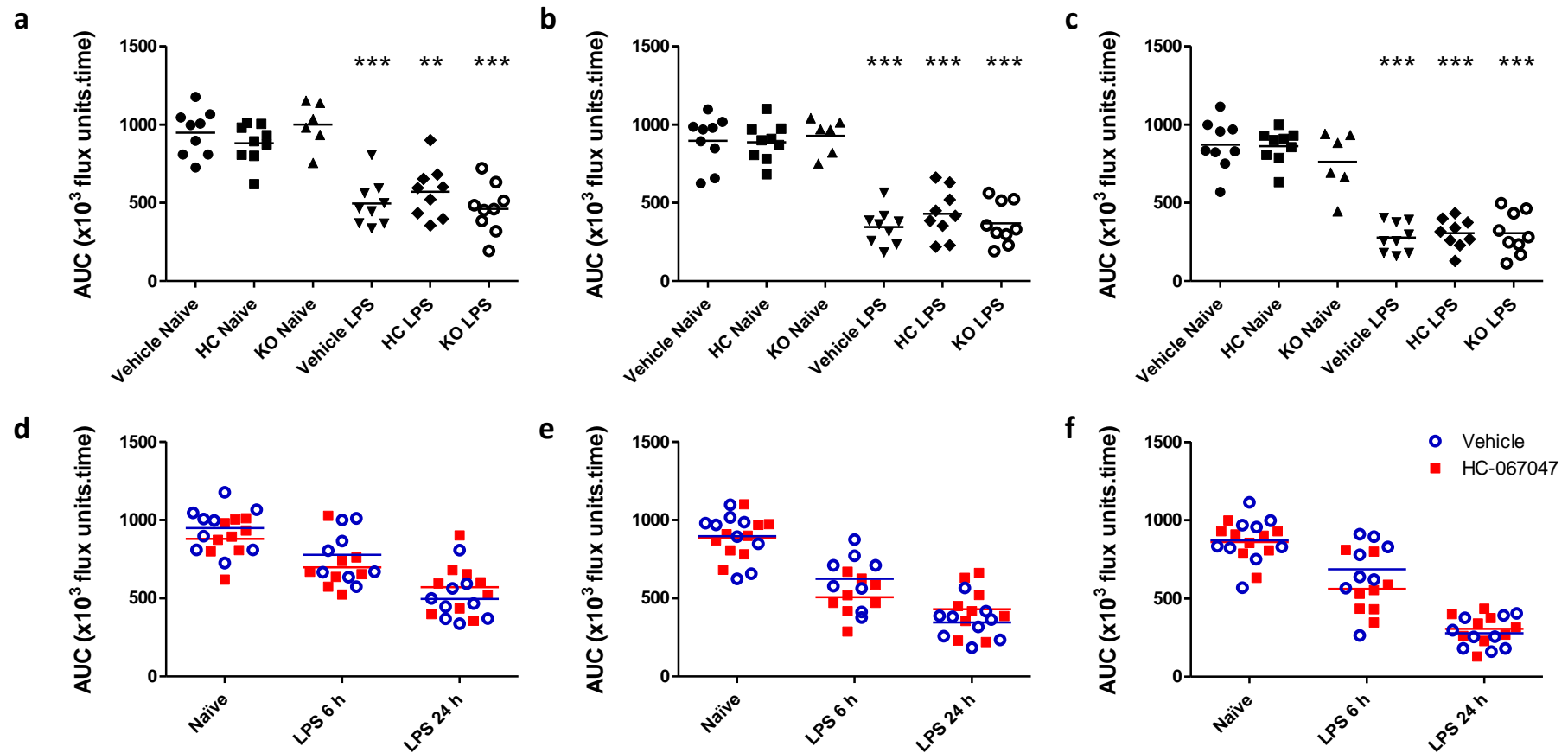


Figure 53. Mesenteric blood flow in healthy and septic TRPV4 WT and KO mice. (a–c) Mesenteric blood flow in TRPV4 KO mice and WT mice treated i.p. with vehicle (10% DMSO) or HC-067047 (HC) for 24 h, either under naïve or septic (LPS 12.5 mg/kg, i.v., 24 h). Data are expressed as total area under the curve (AUC; x10³ flux units.time) over 5-min baseline recording in 1st, 2nd and 3rd order vessels, respectively. **p<0.01, ***p<0.001, relative to naïve counterparts, 1-way ANOVA + Bonferroni post-hoc test. (d–f) Mesenteric blood flow in WT mice treated i.p. with vehicle (10% DMSO) or HC-067047, either under naïve (24-h treatment) or septic conditions (6 h or 24 h; drugs administered simultaneously with LPS) in 1st, 2nd and 3rd order vessels, respectively. AUC for each animal is represented as an individual symbol, with horizontal line denoting group mean.

6.3.5 Neither antagonism nor gene ablation of TRPV4 significantly alters the sepsis phenotype

Antagonism and gene ablation of TRPV4 had only minor effects on clinical phenotype, though in opposing directions. In line with marginally improved mesenteric perfusion, antagonist-treated mice exhibited a slightly lower severity score after 24-h treatment with LPS, relative to vehicle-treated controls. On the other hand, the severity score in TRPV4 KO mice was significantly greater than in antagonist-treated counterparts (**Fig. 54a**). The percentage weight loss over 24 h of sepsis was roughly equivalent between groups, but was lower in antagonist-treated and TRPV4 KO mice, than in vehicle-treated controls (**Fig. 54b**). Core temperature decreased significantly in all LPS-treated mice, though remained stable in all animals throughout the recording period (**Fig. 54c**). Although no statistically significant differences in core temperature were observed between groups, there were trends towards attenuated hypothermia in HC-067047-treated mice, and exaggerated hypothermia in TRPV4 KO mice, relative to vehicle-treated counterparts.

TRPV4 antagonism and gene ablation appeared to have divergent effects on blood biochemistry (**Table 14**). Whereas urea was elevated in all groups 24 h post-LPS, the increase in TRPV4 KO mice was greatest, whereas that in antagonist-treated mice did not reach statistical significance. Blood pH was also lowest in LPS-treated TRPV4 KO mice, whereas acidosis was slightly attenuated in antagonist-treated mice, relative to vehicle-treated controls. PCO₂ was slightly increased by LPS treatment, and bicarbonate slightly decreased, indicative of respiratory and metabolic acidosis, respectively, though no significant differences were observed across treatment groups. All mice also became significantly hypoglycaemic following the induction of sepsis. While both vehicle- and antagonist-treated animals became significantly hypernatraemic and hyperchloraemic over the course of sepsis, consistent with dehydration and loss of bicarbonate, respectively, no change in plasma Na⁺ or Cl⁻ levels was observed in TRPV4 KO mice. Correspondingly, bicarbonate levels decreased in both vehicle- and antagonist-treated mice, but not in TRPV4 KO mice. Both haemoglobin and haematocrit decreased slightly with sepsis, though to an equivalent extent across groups.

Interestingly, like naïve TRPV1 KO animals, naïve TRPV4 KO mice also exhibited trends towards pathological blood biochemistry. Urea was slightly elevated in these mice, and blood pH significantly decreased, relative to vehicle-treated WT controls. Basal haemoglobin and

haematocrit levels were also slightly decreased in these animals, relative to WT controls, indicative of basal anaemia (**Table 14**).

The effect of TRPV4 antagonism was also assessed at an earlier time-point in sepsis pathogenesis. In contrast with the 24-h time-point, no significant difference in severity score, percentage weight loss, or core temperature was observed between vehicle- and antagonist-treated mice at 6 h post-LPS (**Fig. 55**). Blood biochemistry was similarly equivalent between treatment groups at the 6-h time-point (**Table 15**).

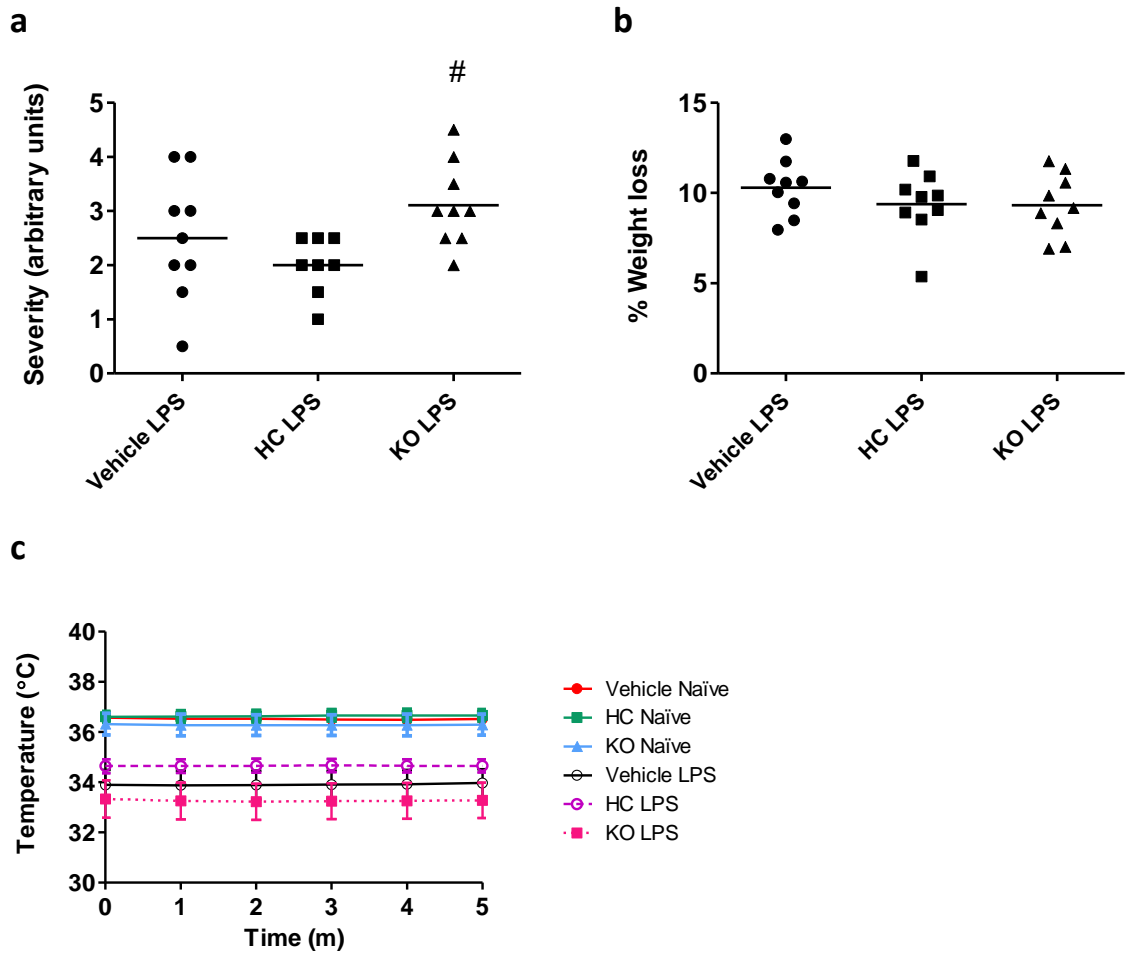


Figure 54. Clinical parameters in TRPV4 WT and KO mice. (a) Arbitrary severity score assigned in a blinded fashion after 24-h treatment with LPS (12.5 mg/kg, i.v.) based voluntary mobility, gait, aversion to touch, facial expression and piloerection. WT mice were treated i.p. with vehicle (10% DMSO) or HC-067047 (HC) at the time of LPS administration. TRPV4 KO mice did not receive any pharmacological treatment. **(b)** Percentage weight loss over 24-h septic period. Values for each animal are presented as individual symbols, with horizontal line denoting group mean. [#] $p < 0.05$, relative to HC-treated animals, 1-wayANOVA + Bonferroni post-hoc test ($n = 8-9$). **(c)** Core temperature measured by rectal probe throughout blood flow recording period. Data are presented as mean \pm SEM ($n = 8-9$).

	Vehicle Naïve	HC Naïve	KO Naïve	Vehicle LPS	HC LPS	KO LPS
Urea (mmol/L)	5.100 ± 0.51	4.938 ± 0.44	5.417 ± 0.39	22.45 ± 5.55*	18.53 ± 3.81	30.30 ± 4.85***
pH	7.314 ± 0.01	7.291 ± 0.01	7.189 ± 0.03 [#]	7.095 ± 0.03***	7.156 ± 0.02**	7.090 ± 0.04
Base excess (mmol/L)	-12.38 ± 0.80	-11.38 ± 1.03	-14.83 ± 0.79	-17.75 ± 1.05	-16.11 ± 1.23	-16.78 ± 2.05
HCO₃⁻ (mmol/L)	13.73 ± 0.88	15.41 ± 1.09	13.55 ± 0.94	11.90 ± 0.73	12.88 ± 1.26	13.14 ± 1.74
PCO₂ (mmHg)	27.25 ± 2.18	32.06 ± 2.40	36.65 ± 5.20	39.08 ± 3.15	36.90 ± 4.02	42.11 ± 4.99
TCO₂ (mmol/L)	14.63 ± 0.92	16.50 ± 1.15	14.67 ± 1.02	13.13 ± 0.74	14.00 ± 1.36	14.33 ± 1.85
Glucose (mmol/L)	11.70 ± 1.03	12.36 ± 0.90	10.77 ± 1.10	1.08 ± 0.04***	1.34 ± 0.12***	1.76 ± 0.50***
Na⁺ (mmol/L)	150.3 ± 1.7	147.9 ± 1.0	150.2 ± 0.9	155.9 ± 0.9*	154.0 ± 1.1**	150.6 ± 1.0 [#]
K⁺ (mmol/L)	4.088 ± 0.15	4.250 ± 0.19	4.150 ± 0.24	3.950 ± 0.30	3.589 ± 0.32	3.625 ± 0.23
Cl⁻ (mmol/L)	121.8 ± 1.3	119.5 ± 0.8	124.0 ± 0.6	130.0 ± 1.6*	128.6 ± 1.0***	124.3 ± 1.6 [#]
Anion gap (mmol/L)	18.50 ± 0.53	17.25 ± 0.94	16.67 ± 0.92	18.50 ± 0.87	16.11 ± 1.11	16.25 ± 1.03
Haemoglobin (g/dl)	10.41 ± 0.54	10.93 ± 0.59	8.60 ± 0.25	9.43 ± 0.71	8.96 ± 7.33	9.53 ± 0.59
Haematocrit (%PCV)	30.63 ± 1.60	32.13 ± 1.74	25.25 ± 0.75	29.57 ± 1.17	27.88 ± 1.36	28.00 ± 1.72

Table 14. Blood biochemistry in healthy and septic TRPV4 WT and KO mice. Blood gas and biochemistry were measured from venous blood samples by iSTAT point-of-care analyser in either TRPV4 KO mice, or WT mice treated i.p. with vehicle (10% DMSO) or HC-067047 (HC) for 24 h, either under naïve or septic (LPS 12.5 mg/kg, i.v., 24 h) conditions. Data are presented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, relative to naïve controls; [#]p<0.05, relative to vehicle-treated controls, 1-way ANOVA + Bonferroni post-hoc test (n = 6–9).

Abbreviations: PCO₂, partial pressure of CO₂; TCO₂, total carbon dioxide; PCV, packed cell volume; HC, HC-067047; KO, TRPV4 knockout; LPS, lipopolysaccharide

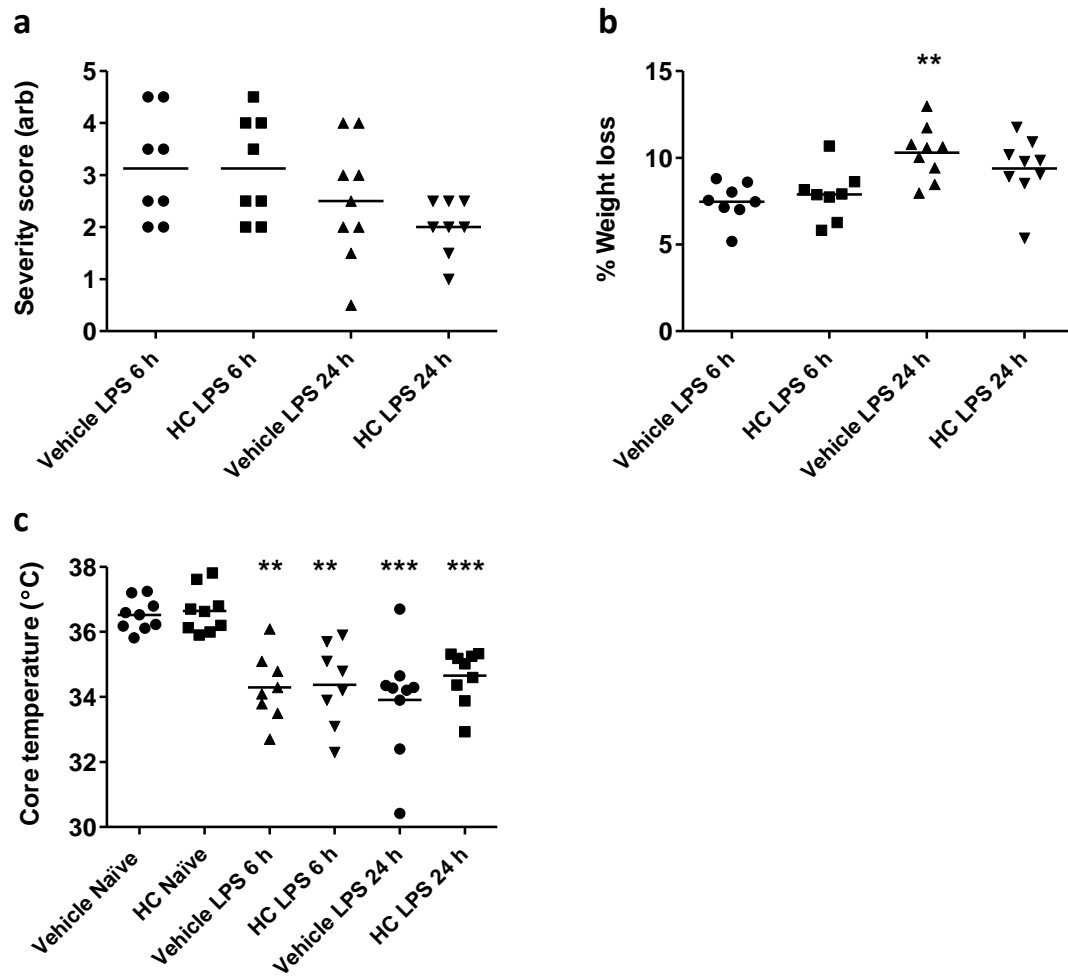


Figure 55. Clinical parameters in WT mice treated with TRPV4 antagonist or vehicle. (a) Arbitrary severity score assigned in a blinded fashion after 6- or 24-h treatment with LPS (12.5 mg/kg, i.v.) based voluntary mobility, gait, aversion to touch, facial expression and piloerection. WT mice were treated i.p. with vehicle (10% DMSO) or HC-067047 (HC) at the time of LPS administration **(b)** Percentage weight loss over 6- or 24-h septic period. **(c)** Mean core temperature measured by rectal probe over blood flow recording period. Values for each animal are presented as individual symbols, with horizontal line denoting group mean. ** $p < 0.01$, *** $p < 0.001$, relative to naïve controls, 1-wayANOVA + Bonferroni post-hoc test ($n = 8-9$).

	Vehicle Naïve	HC Naïve	Vehicle LPS 6 h	HC LPS 6 h	Vehicle LPS 24 h	HC LPS 24 h
Urea (mmol/L)	5.100 ± 0.51	4.938 ± 0.44	7.888 ± 0.51	7.838 ± 0.76	22.45 ± 5.55*	18.53 ± 3.81
pH	7.314 ± 0.01	7.291 ± 0.01	7.073 ± 0.03***	7.090 ± 0.02***	7.095 ± 0.03***	7.156 ± 0.02**
Base excess (mmol/L)	-12.38 ± 0.80	-11.38 ± 1.03	-19.00 ± 1.18*	-18.63 ± 1.16**	-17.75 ± 1.05	-16.11 ± 1.23
HCO₃⁻ (mmol/L)	13.73 ± 0.88	15.41 ± 1.09	11.09 ± 0.89	11.30 ± 1.13	11.90 ± 0.73	12.88 ± 1.26
PCO₂ (mmHg)	27.25 ± 2.18	32.06 ± 2.40	38.25 ± 3.28	37.46 ± 4.41	39.08 ± 3.15	36.90 ± 4.02
TCO₂ (mmol/L)	14.63 ± 0.92	16.50 ± 1.15	12.13 ± 0.97	12.38 ± 1.22	13.13 ± 0.74	14.00 ± 1.36
Glucose (mmol/L)	11.70 ± 1.03	12.36 ± 0.90	2.063 ± 0.52***	1.838 ± 0.42***	1.08 ± 0.04***	1.34 ± 0.12***
Na⁺ (mmol/L)	150.3 ± 1.7	147.9 ± 1.0	156.8 ± 0.4**	156.6 ± 0.5***	155.9 ± 0.9*	154.0 ± 1.1**
K⁺ (mmol/L)	4.088 ± 0.15	4.250 ± 0.19	3.788 ± 0.20	3.788 ± 0.33	3.950 ± 0.30	3.589 ± 0.32
Cl⁻ (mmol/L)	121.8 ± 1.3	119.5 ± 0.8	131.3 ± 1.0***	131.4 ± 0.9***	130.0 ± 1.6*	128.6 ± 1.0***
Anion gap (mmol/L)	18.50 ± 0.53	17.25 ± 0.94	18.13 ± 1.11	17.50 ± 0.50	18.50 ± 0.87	16.11 ± 1.11
Haemoglobin (g/dl)	10.41 ± 0.54	10.93 ± 0.59	9.44 ± 0.64	9.23 ± 0.85	9.43 ± 0.71	8.96 ± 7.33
Haematocrit (%PCV)	30.63 ± 1.60	32.13 ± 1.74	27.75 ± 1.92	29.14 ± 1.71	29.57 ± 1.17	27.88 ± 1.36

Table 15. Blood biochemistry in healthy and septic WT mice treated with TRPV4 antagonist or vehicle. Blood gas and biochemistry were measured from venous blood samples by iSTAT point-of-care analyser in WT mice treated i.p. with vehicle (10% DMSO) or HC-067047 (HC), either under naïve (24-h treatment) or septic (LPS 12.5 mg/kg, i.v., 6 h or 24 h) conditions. Data are presented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, relative to naïve controls, 1-way ANOVA + Bonferroni post-hoc test (n = 8–9).

Abbreviations: PCO₂, partial pressure of CO₂; TCO₂, total carbon dioxide; PCV, packed cell volume; HC, HC-067047; KO, TRPV4 knockout; LPS, lipopolysaccharide

6.3.6 TRPV4 KO mice exhibit exaggerated kidney, liver and spleen oedema in LPS-induced sepsis

Oedema formation was measured by comparing wet and dry weights of various tissues. Surprisingly, treatment with LPS for 24 h did not cause any appreciable oedema formation in WT mice (**Fig. 56**). Septic TRPV4 KO mice exhibited marginally elevated oedema formation in the liver and spleen, and significantly increased oedema in the kidney. No difference was observed between vehicle and antagonist-treated mice.

The effect of TRPV4 antagonism on oedema formation was also assessed at an earlier time-point in sepsis pathogenesis. In contrast with the 24-h time-point, significant liver oedema was observed in both vehicle- and HC-067047-treated mice after 6 h, though this had returned to baseline levels by 24 h post-LPS (**Fig. 57**). No significant difference in oedema formation was observed in any other tissues, however. No changes in the ratio of heart weight to body weight were observed following the induction of sepsis in any of the animal groups.

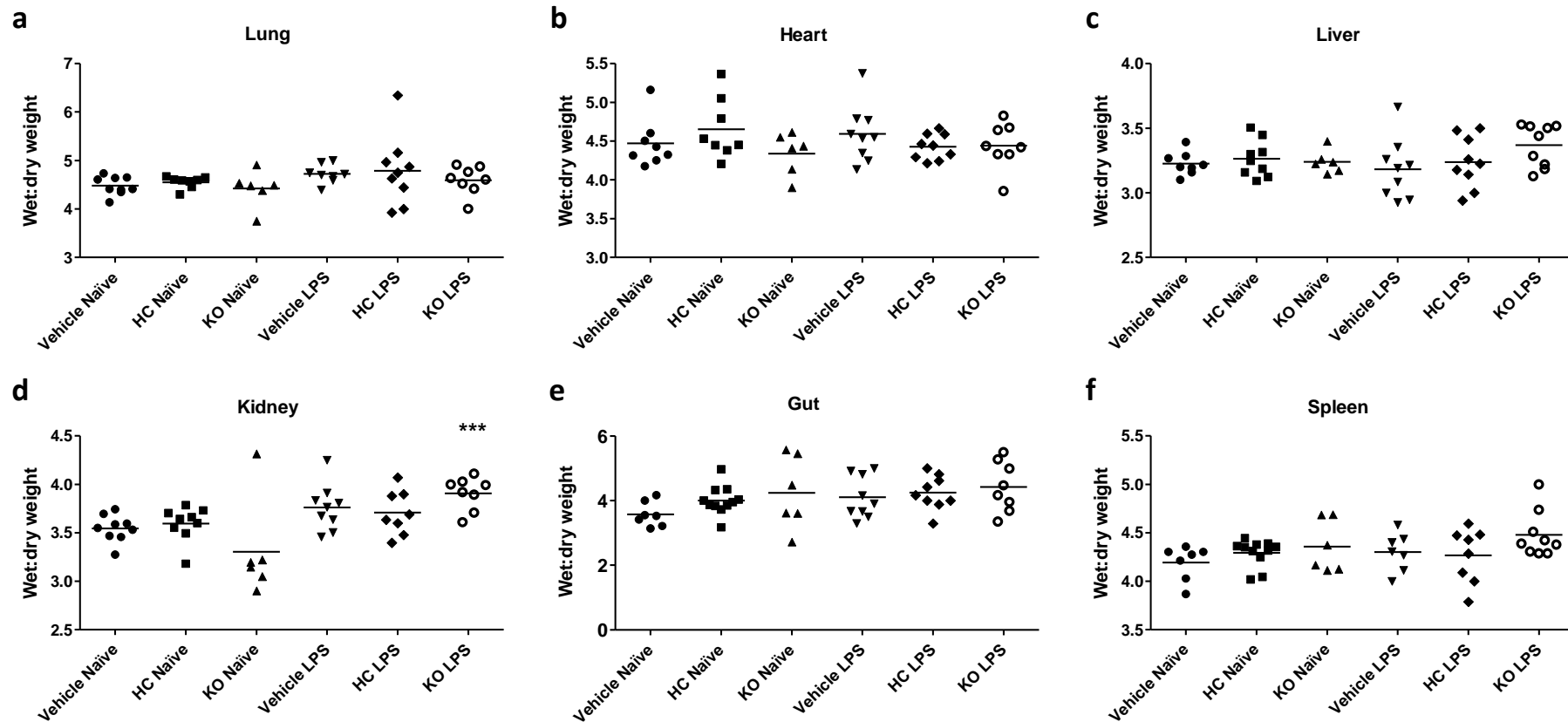


Figure 56. Oedema formation in TRPV4 WT and KO mice. Oedema formation was measured by comparing wet and dry weights of **(a)** lungs, **(b)** hearts, **(c)** upper liver lobes, **(d)** right kidneys, **(e)** 1-cm sections of small intestine and **(f)** whole spleens. WT mice treated i.p. with vehicle (10% DMSO) or HC-067047 (HC) for 24 h, either under naïve or septic (LPS 12.5 mg/kg, i.v., 24 h) conditions. Values for each animal are presented as individual symbols, with horizontal line denoting group mean. *** $p < 0.001$, relative to naïve controls, 1-wayANOVA + Bonferroni post-hoc test ($n = 6-9$).

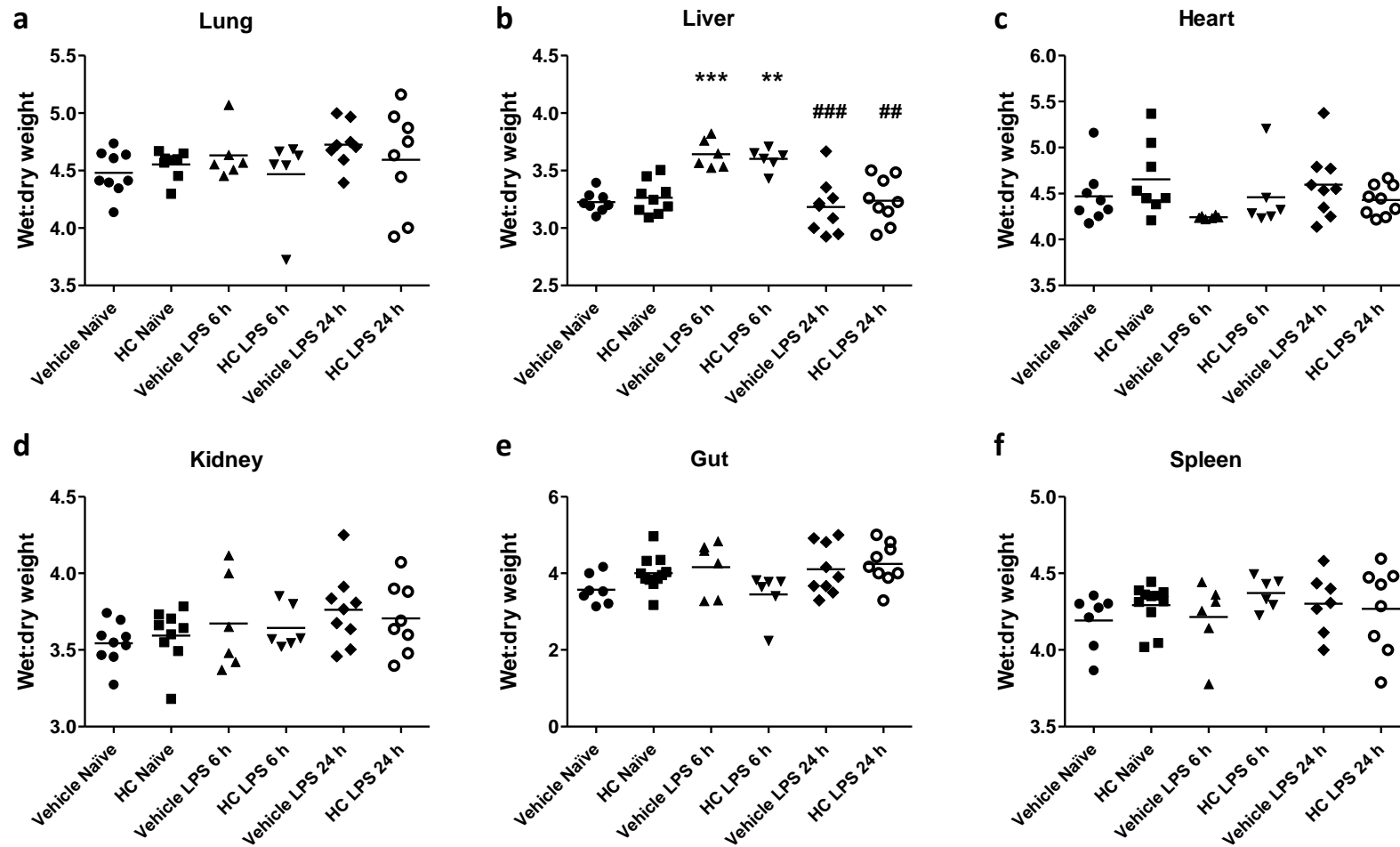


Figure 57. Oedema formation in WT mice treated with TRPV4 antagonist or vehicle. Oedema formation was measured by comparing wet and dry weights of (a) lungs, (b) hearts, (c) upper liver lobes, (d) right kidneys, (e) 1-cm sections of small intestine and (f) whole spleens. WT mice were treated i.p. with vehicle (10% DMSO) or HC-067047 (HC) at the time of LPS (12.5 mg/kg, i.v., 6 or 24 h). Values for each animal are presented as individual symbols, with horizontal line denoting group mean. ** $p < 0.01$, *** $p < 0.001$, relative to naïve controls, 1-wayANOVA + Bonferroni post-hoc test ($n = 6-9$).

6.3.7 Vasoactive responses to topical TRPV4 activation are enhanced in sepsis

In order to assess changes in TRPV4 activity *in vivo* after the induction of sepsis, 1 μ M GSK1016790A and vehicle (2% DMSO in pre-warmed saline) were sequentially applied to the exposed mesenteric bed by aerosolised spray. No significant responses to GSK1016790A were observed in the mesenteric beds of naive mice: both vehicle and GSK1016790A caused a slight non-significant decrease in blood flow, likely to reflect evaporative cooling (**Fig. 58a–c** and **Table 16**). In LPS-treated mice, on the other hand, GSK1016790A caused a modest increase in blood flow that was significant in 2nd and 3rd order mesenteric vessels (**Fig. 58d–f** and **Table 16**).

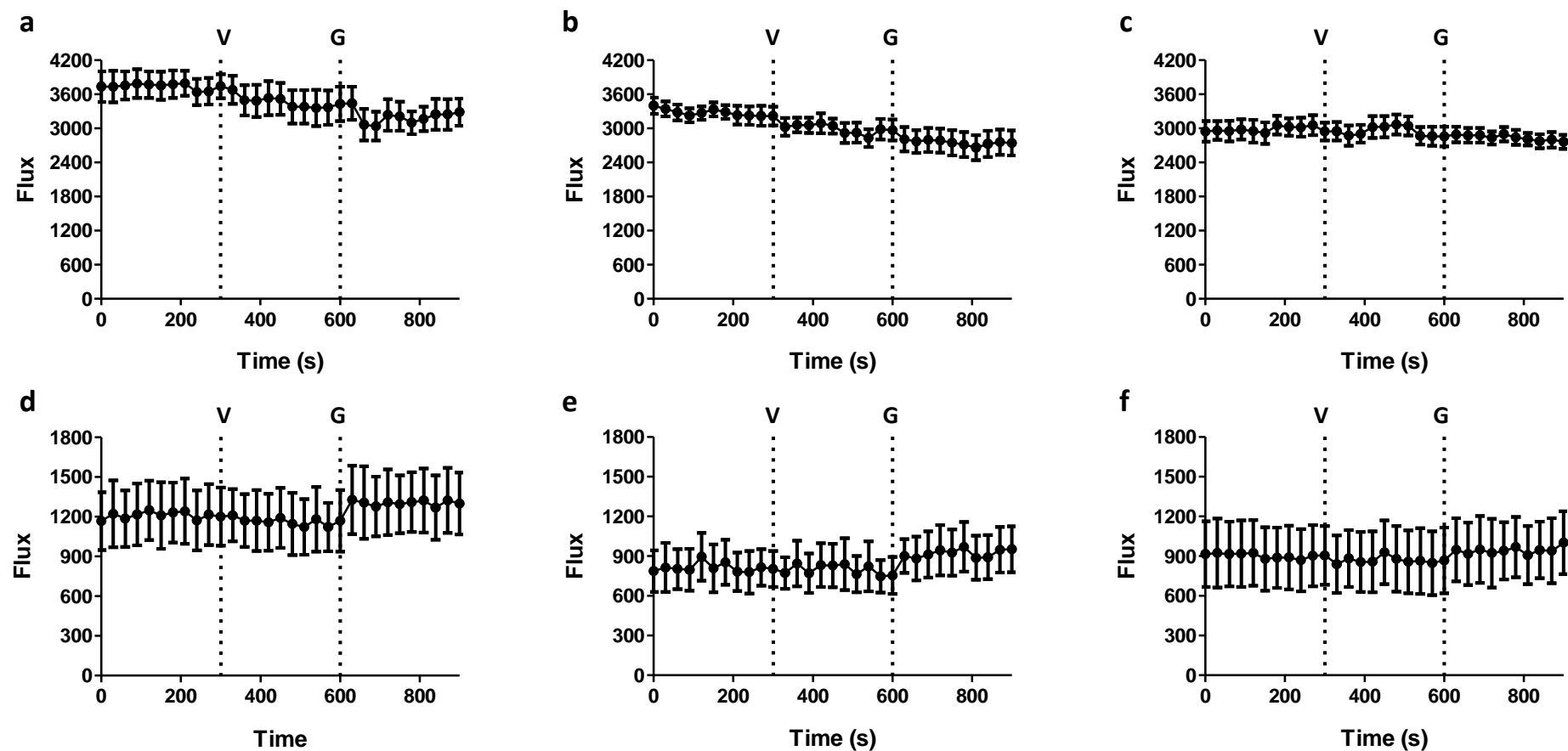


Figure 58. Vasoactive responses to GSK1016790A and vehicle in healthy and septic WT mice. Baseline mesenteric blood flow was recorded for 5 min in naïve (a–c) and septic (d–f) mice, 24 h after injections of LPS. Vehicle (V; 2% DMSO in saline) was then administered as an aerosolised spray, followed by administration of GSK1016790A (G; 1 μ M) 5 min later. Blood flow was recorded in (a & d) 1st order, (b & e) 2nd order and (c & f) 3rd order mesenteric vessels. Data are presented as mean \pm SEM. * p <0.05, area under the curve relative to baseline, 1-way ANOVA + Bonferroni post-hoc test (n = 5).

	Naive			LPS 24 h		
	<i>1st order</i>	<i>2nd order</i>	<i>3rd order</i>	<i>1st order</i>	<i>2nd order</i>	<i>3rd order</i>
BL-Veh	-7.33 ± 3.10%	-8.39 ± 1.73%	-1.10 ± 0.32%	-3.00 ± 2.77%	-1.18 ± 1.51%	-2.22 ± 3.87%
Veh-GSK	-7.61 ± 1.30%	-8.46 ± 3.27%	-3.42 ± 2.05%	+12.11 ± 6.19%	+14.71 ± 6.70% ^{*,#}	+11.73 ± 8.72% ^{**}

Table 16. Change in mesenteric blood flow following topical TRPV4 activation in naïve and septic mice. Baselines (BL), and responses to aerosolised vehicle (Veh; 10% DMSO in pre-warmed saline) and GSK1016790A (GSK; 1 µM) were recorded over sequential 5-min periods. Data are presented as mean percentage change in area under the curve over 5-min recording period. *p<0.05, **p<0.01, relative to vehicle response in the same mouse, paired 2-tailed Student's T-test; [#]p<0.05 relative to corresponding response in naïve controls, 1-way ANOVA + Bonferroni post-hoc test (n = 5).

6.4 Summary

The key findings from this study are:

1. Loss of TRPV4 activity is associated with slightly reduced water intake
2. TRPV4 KO mice have normal basal haemodynamic function, but exhibit a trend towards lower blood pressure and reduced ambulatory activity
3. Systemic TRPV4 antagonism caused a modest and transient increase in blood pressure, followed by a subsequent decrease in night-time blood pressure, though systemic administration of vehicle produced similar effects, albeit of lower magnitude
4. Loss of TRPV4 activity may be associated with marginally exaggerated hypotension in sepsis, though a larger sample size would be needed to confirm this observation
5. TRPV4 does not appear to play a significant role in the regulation of mesenteric blood flow, either under basal or septic conditions
6. TRPV4 antagonism slightly attenuates the clinical phenotype of sepsis, whereas TRPV4 gene ablation is associated with a slightly exaggerated phenotype
7. Topical TRPV4 activation in the mesenteric bed does not cause significant changes in blood flow under naïve conditions, but slightly increases blood flow in septic mice

6.5 Discussion

Based on our own observations of LPS-induced sensitisation of endothelial TRPV4 (**Chapter 5**), and in light of previous studies demonstrating that excessive TRPV4 activation can cause profound hypotension, endothelial failure and circulatory collapse (Willette et al., 2008), we hypothesised that TRPV4 may contribute to haemodynamic and vascular dysfunction during sepsis. Numerous endogenous agonists and regulators of TRPV4, including anandamide (Varga et al., 1998), arachidonic acid and its metabolites (Bruegel et al., 2012), and PKA (Yang et al., 1997), have been shown to be upregulated in sepsis. It is conceivable therefore, that inflammation-induced upregulation of endogenous factors could contribute to sepsis-associated circulatory failure via excessive TRPV4 activation. By extension, blockade of TRPV4 activity may be expected to attenuate the circulatory dysfunction that underlies sepsis pathogenesis. To test this hypothesis we used both TRPV4 KO mice and a selective TRPV4 antagonist.

6.5.1 Basal phenotype of mice lacking TRPV4

In contrast to the parent colony housed at the Wolfson Centre for Age-Related Diseases, King's College London, heterozygous mice re-derived from frozen embryos in our animal unit did not produce TRPV4 KO mice in the expected Mendelian ratios: only 5 male TRPV4 KOs were born following multiple rounds of breeding by 8 heterozygous pairs. Mice lacking TRPV4 have been shown to be fully viable (Liedtke and Friedman, 2003), and the fact that some KOs were generated in this study demonstrate that the mutation is not developmentally lethal. It is unclear, therefore, why the yield of TRPV4 KO mice was so low. It is possible that epigenetic changes acquired during embryo isolation, freezing, transport or implantation could have contributed to *in utero* selection against mutant zygotes, and that homozygous crosses of TRPV4 KO mice would be required to generate sufficient numbers of mutant mice. This was unfortunately not possible for this study, due to time constraints, and experiments were thus limited by the small sample size. Therefore meaningful statistical comparison was only possible for antagonist-treated mice, though trends in KO mice support further investigation.

Consistent with a role for TRPV4 in osmoregulation, and with previous reports (Liedtke and Friedman, 2003), we observed a modest decrease in water intake in TRPV4 KO mice. Meaningful comparison was limited by the low mouse number, although it is supported by the observation that systemic treatment with a TRPV4 antagonist also reduced water intake over the following 24 h. Previous reports suggest that differences in water intake between mutant and WT mice are only evident when mice are singly housed, as they were in this study: mutually-stimulating group drinking patterns and free access to food both increase water

intake and mask genotype differences (Liedtke and Friedman, 2003). The decrease in water intake observed in this study is in contrast with a previous report which recorded no differences in water intake between HC-067047- and vehicle-treated mice (Everaerts et al., 2010). Differences in the composition of the vehicle (1% DMSO versus 10% in this study) could account for this discrepancy; it is also possible the differences observed here would have been further enhanced in the absence of food. The observation that food intake is unaffected by loss of TRPV4 activity suggests that the decrease in water intake represents a reduced desire to drink, rather than a non-specific physiological effect that indirectly alters consumption behaviour.

6.5.2 The role of TRPV4 in basal haemodynamic regulation

Consistent with previous reports (Zhang et al., 2009, Nishijima et al., 2014), TRPV4 KO mice exhibited a trend towards lower basal arterial pressure during both daytime and night-time periods. This is surprising given that TRPV4 has been implicated in both NO- and EDHF-mediated vasodilatory responses to physical and chemical stimuli in both conduit and resistance arteries (Kohler et al., 2006, Hartmannsgruber et al., 2007, Loot et al., 2008, Zhang et al., 2009, Mendoza et al., 2010, Adapala et al., 2011, Rath et al., 2012, Sonkusare et al., 2012, Ma et al., 2013a). A possible explanation is that loss of TRPV4 leads to compensatory upregulation of alternative vasodilatory pathways during development; although studies of isolated vessels from TRPV4 KO mice have shown impaired vasodilatation in response to a range of physiological stimuli (Zhang et al., 2009, Earley et al., 2009), the effects of isolation and *ex vivo* culture are unknown. Alternatively, it is possible that loss of TRPV4 expression in vascular smooth muscle may represent loss of a vasoconstrictor mechanism. While numerous studies have suggested that TRPV4 in vascular smooth muscle couples with hyperpolarising K⁺ channels to induce vasodilatation (Earley et al., 2005, Earley et al., 2009), global activation of TRPV4 (as opposed to very localised activation) may trigger Ca²⁺-dependent vasoconstriction that may contribute to the maintenance of vascular tone. Indeed, TRPV4-mediated vasoconstriction has previously been demonstrated in the lung, where TRPV4 gene ablation suppresses the development of chronic hypoxic pulmonary hypertension (Xia et al., 2013).

Given that TRPV4 exhibits tonic activity both in heterologous expression systems (Strotmann et al., 2000) and in primary endothelial cells (Sonkusare et al., 2012), its blockade may be expected to cause acute hypertension. While systemic administration of HC-067047 did induce a small transient increase in blood pressure under basal conditions, a similar effect was observed in vehicle-treated animals, suggesting that the transient hypertension may have been a stress response to manual handling. In accordance with these data, administration of a

different TRPV4 antagonist – GSK 2193874 – did not significantly alter blood pressure after either acute intravenous administration or repeated oral gavage (Thorneloe et al., 2012)

The lack of any dramatic effect on blood pressure is consistent with a previous *ex vivo* report that blockade of the TRPV4-CYP450 epoxygenase axis only inhibits flow-induced vasodilatation in the presence of eNOS and prostacyclin antagonists (Loot et al., 2008). In other words, blockade of one endothelium-dependent vasodilatory pathway can lead to acute upregulation of a compensatory pathway. Upregulation of alternative (and perhaps slightly more potent) endothelial vasodilatory pathways following loss of TRPV4 activity may also explain the subsequent night-time decreases in blood pressure following HC-067047 administration, which were not evident in vehicle-treated mice (as well as the trend towards hypotension in TRPV4 KO mice). A compensatory increase in eNOS activity and PGI₂ production could also account for the night-time decreases in heart rate following TRPV4 antagonism, since both are known to confer negative chronotropy (Feron et al., 1998, Chiba and Malik, 1980).

6.5.3 Haemodynamic consequences of TRPV4 deletion during sepsis

While the haemodynamic role of TRPV4 has been studied under physiological conditions (Willette et al., 2008, Gao et al., 2009, Zhang et al., 2009, Gao and Wang, 2010, Thorneloe et al., 2012), its function in pathological and inflammatory settings has not been well characterised. Based on our own observations of LPS-induced sensitisation of endothelial TRPV4 (**Chapter 5**), and in light of previous studies demonstrating that excessive TRPV4 activation can cause profound hypotension, endothelial failure and circulatory collapse (Willette et al., 2008), we hypothesised that TRPV4 may contribute to haemodynamic and vascular dysfunction during sepsis. Numerous endogenous agonists and regulators of TRPV4, including anandamide (Varga et al., 1998), arachidonic acid and its metabolites (Bruegel et al., 2012), and PKA (Yang et al., 1997), have been shown to be upregulated in sepsis. It is conceivable therefore, that inflammation-induced upregulation of endogenous factors could contribute to sepsis-associated circulatory failure via excessive TRPV4 activation. We hypothesised that blockade of TRPV4 activity would attenuate the circulatory dysfunction that occurs in sepsis.

Surprisingly, neither TRPV4 gene deletion nor pharmacological antagonism attenuated the LPS-induced haemodynamic dysfunction. In fact, both HC-067047-treated TRPV4 KO mice exhibited a trend towards exaggerated hypotension that was most evident in measurements of systolic pressure. The exaggerated systolic hypotension and greater decrease in pulse pressure observed in both antagonist-treated and KO mice may be indicative of reduced cardiac output and left ventricular dysfunction, though these parameters were not measured in this study.

Whether reduced water intake prior to the induction of sepsis may have contributed to exaggerated hypovolaemia is unclear. Alternatively, it is possible that elevated NO production (as a response to TRPV4 loss) could have increased aortic compliance in these animals, leading to greater systolic hypotension and a narrower pulse pressure.

Overall, the haemodynamic phenotype of sepsis appeared to be worse in mice lacking TRPV4: hypotension was slightly exaggerated, and the baroreceptor reflex was almost entirely absent in TRPV4 KO mice. Given that NO is thought to antagonise the positive chronotropic effects of increased sympathetic drive (Feron et al., 1998), it is possible that excessive NO production in TRPV4 KO mice could account for the pronounced bradycardia following LPS administration. It will be interesting, in the future, to compare tissue levels of eNOS and iNOS, and plasma levels of NO and PGI₂ in mice with and without functional TRPV4, both under basal and septic conditions.

6.5.4 TRPV4 in microvascular regulation

Despite numerous reports of an important role for TRPV4 in mediating agonist- and flow-induced vasoactive responses in small resistance vessels (Kohler et al., 2006, Earley et al., 2009, Zhang et al., 2009, Mendoza et al., 2010, Adapala et al., 2011, Sonkusare et al., 2012, Ma et al., 2013a), we did not observe any marked changes in mesenteric blood flow in antagonist-treated or TRPV4 KO mice. While there was a trend towards slightly improved flow in 1st and 2nd order vessels in septic HC-067047-treated mice, this is unlikely to represent a true biological phenomenon as it was not evident in TRPV4 KO mice. Of note, perhaps, is the observation that antagonist-treated mice exhibited trends towards exaggerated hypotension following LPS administration, but marginally better mesenteric perfusion; these results are consistent with our hypothesis put forward in **Chapter 3**, that stabilisation of arterial pressure during sepsis may occur at the expense of the microcirculation.

Because the exact half-life of HC-067047 has not been reported, though plasma levels are known to exceed the IC₅₀ for some hours after i.p. administration (Everaerts et al., 2010), we also investigated the effect of TRPV4 antagonism on mesenteric blood flow at an earlier time-point. At 6-h post-LPS, there was a trend towards lower blood flow in antagonist-treated mice, consistent with a vasodilatory role for TRPV4, though this was not significant.

While TRPV4 is known to dilate small resistance vessels (such that its blockade may be expected to decrease mesenteric blood flow) its activation has also been associated with increased endothelial permeability and oedema formation, which would impair perfusion (Willette et al., 2008). These divergent effects on microvascular blood flow may account for

the reverse in trends between 6-h and 24-h treatment with LPS. Nonetheless, our data do not support a prominent role for TRPV4 in regulating mesenteric blood flow in this model, either under basal or septic conditions. It is likely that compensatory pathways are upregulated both acutely and chronically to compensate for its loss.

In contrast to numerous studies demonstrating TRPV4-mediated dilatation of mesenteric arteries (Kohler et al., 2006, Earley et al., 2009, Zhang et al., 2009, Mendoza et al., 2010, Adapala et al., 2011, Sonkusare et al., 2012, Ma et al., 2013a) topical activation of TRPV4 with GSK1016790A did not induce any marked changes in blood flow in naïve mice. Despite the relatively high concentration used (1 μ M), it is possible that owing to the aerosolised delivery method, which may be prone to evaporation, insufficient concentrations of the agonist were able to come into contact with vascular receptors. While TRPV4 is known to be expressed in vascular smooth muscle (Earley et al., 2005), the compound may not have been able to reach the luminal side of the vessel to induce endothelium-dependent vasodilatation. Further experiments using higher concentrations or a different delivery method are required to elucidate this further. The observation that GSK1016790A caused a small increase in blood flow under in LPS-treated mice support our hypothesis that TRPV4 may become sensitised under inflammatory conditions.

6.5.5 TRPV4 in blood biochemistry

Like TRPV1 KO animals, TRPV4 KO mice exhibited trends towards basal pathology. Blood pH was significantly lower in these animals compared with WT counterparts; base excess was correspondingly reduced, and partial pressure of CO₂ elevated, indicative of respiratory acidosis. These data are consistent with a potential acid-sensing role for TRPV4 reported previously (Suzuki et al., 2003). However, because the naïve TRPV4 KO mice used in this study were derived from a separate colony, were measured in a different animal unit, and furthermore were not age-matched, results should be interpreted with caution. Future research comparing the basal phenotype of WT and TRPV4 KO littermates would be required to establish whether loss of TRPV4 is truly associated with basal acidosis. Nonetheless, the observation that blood pH was also slightly reduced in naïve HC-067047-treated mice relative to vehicle-treated controls, suggests that TRPV4 may be involved in maintaining acid-base homeostasis.

Following the induction of sepsis, TRPV4 KO mice exhibited a more dramatic increase in plasma urea concentration than WT mice, consistent with a role for TRPV4 in regulating renal function. Although not measured in this study, it is possible that loss of TRPV4 activity may result in impaired renal circulation. While TRPV4 KO mice also exhibited the lowest blood pH of

all groups in sepsis, however, this was not significantly different from basal pH in these animals (which was mildly acidotic). Both antagonist- and vehicle-treated WT mice, in contrast, exhibited a significant decline in pH between basal and septic conditions, though the magnitude of the decrease was less pronounced in HC-067047-treated animals. This suggests that blockade of TRPV4 activity may attenuate the development of metabolic acidosis. Consistent with this notion, base excess was slightly lower and bicarbonate levels slightly higher in both antagonist-treated and TRPV4 KO mice, relative to vehicle-treated controls.

Both vehicle- and antagonist-treated WT animals became severely hyperchloraemic over the course of sepsis. Fluid loss, and perhaps the administration of saline resuscitation at the time of sepsis, could have contributed to this finding. Hyperchloraemic metabolic acidosis (in which serum Cl^- levels are elevated, bicarbonate reduced and blood pH is low) is also indicative of kidney dysfunction (Walker, 1990). Surprisingly, given the elevated plasma urea levels, TRPV4 KO mice did not exhibit LPS-induced hyperchloraemia or hypernatraemia. Because naïve controls were not properly matched it is difficult to say whether ion concentrations change between physiological and pathological settings, however.

6.5.6 Measurement of oedema formation

Following reports linking excessive TRPV4 activation to endothelial failure and oedema formation (Alvarez et al., 2006, Willette et al., 2008) we aimed to establish the role of TRPV4 in oedema formation during sepsis – a condition characterised by increased endothelial permeability (Bridges and Dukes, 2005). No marked changes in the ratio of tissue wet-to-dry weight were observed in WT mice following 24-h treatment with LPS: only the lung and the kidney exhibited trends towards increased plasma extravasation, and values were not different between vehicle- and antagonist-treated mice. Whether these data indicate that oedema formation is not significant at this time-point in sepsis pathogenesis is not clear; it is possible that the method used is insufficiently sensitive to detect small changes in tissue water volume, and that assessment of Evan's Blue extravasation would have yielded more information.

We did observe evidence of exaggerated oedema formation in the kidneys of LPS-treated TRPV4 KO mice (there were also trends towards increased wet-to-dry weight ratios in the livers and spleens of these animals). This was surprising given that these mice did not exhibit an altered ionic balance, and furthermore, because activation of TRPV4 has been associated with increased permeability (Alvarez et al., 2006, Willette et al., 2008). Nonetheless, it appears that in our hands deletion of the TRPV4 gene is associated with slightly exaggerated oedema formation in the kidney, liver and spleen following LPS administration, though it is not clear what mechanism may be involved.

6.5.7 *In vivo* administration of HC-067047

One possible explanation for the lack of any dramatic effect following treatment with HC-067047 in either naïve or septic animals is inadequate dosing. The dose of HC-067047 used in this study (10 mg/kg) was chosen based on the only two previous reports of its administration *in vivo* (Everaerts et al., 2010, Materazzi et al., 2012). While both previous studies used vehicle concentrations of DMSO significantly lower than the one used here (1% and 2.5%, respectively), in this study, 10% DMSO was found to represent the lowest concentration in which HC-067047 was completely soluble: at lower concentrations white particulate matter was clearly visible in the solution. Whether lower solubility may account for the lack of differences in water intake in HC-067047-treated mice reported previously (Everaerts et al., 2010) is unclear. It is equally possible that certain unknown environmental factors (ambient temperature, purity of DMSO) could have affected the solubility of HC-067047 in this study.

While the effects of systemic TRPV4 antagonism were not dramatic in this study, use of higher concentrations was not possible: intraperitoneal injection of 100 mg/kg HC-067047 has been shown to cause obvious adverse effects, including hunching and piloerection (Everaerts et al., 2010), and higher concentrations of DMSO could potentially induce cellular toxicity. Repeated dosing may represent a more viable option for blood flow recording or biochemical analysis, though repeated manual handling and intraperitoneal injections would certainly preclude meaningful haemodynamic recording. Supplementation of drinking water with orally available TRPV4 antagonists (Thorneloe et al., 2012) would be required to elucidate further the effects of TRPV4 blockade on cardiovascular function in sepsis.

Nonetheless, plasma levels of the compound have been shown to remain above the IC₅₀ for more than 2 hours after intraperitoneal administration of 10 mg/kg HC-067047 (Everaerts et al., 2010), suggesting that any prominent role for TRPV4 in sepsis-associated cardiovascular dysfunction would at least have been evident in the early phase of sepsis. The fact that no major changes in haemodynamics, mesenteric blood flow, blood biochemistry or tissue oedema were observed at 6 h post-LPS, suggests either that TRPV4 does not play a major role in cardiovascular regulation during sepsis or that other pathways can be upregulated acutely to compensate for its loss. However, as the TRPV4 KO study was underpowered, and the antagonist was only administered as a single dose, further studies in which research conditions are optimised may yet uncover evidence of a vasoregulatory role for TRPV4 in sepsis.

6.5.8 Concluding remarks

Overall, our data do not support a major role for TRPV4 in sepsis-associated cardiovascular dysfunction. It is possible that upregulation of alternative endothelium-dependent vasodilatory pathways may compensate for its loss both acutely and chronically, and that changes would only be evident on blockade of NO- and PGI₂-dependent pathways. Alternatively, since plasma osmolality is known to increase in sepsis (Jochberger et al., 2009), and hypertonicity inhibits TRPV4 activity (Strotmann et al., 2000), it is possible that under septic conditions, TRPV4 activity is inhibited such that gene deletion or pharmacological antagonism will produce no further effect. Regardless of underlying mechanisms, based on data obtained in this study, TRPV4 does not appear to be a viable target in the treatment of sepsis-associated cardiovascular dysfunction.

CHAPTER 7 | GENERAL DISCUSSION

7.1 Overview

Sepsis is an overwhelming inflammatory response to infection that can progress to septic shock, characterised by refractory hypotension and insufficient organ perfusion. It is associated with significant global morbidity and mortality, accounting for almost 40,000 deaths per annum in the UK alone (Daniels, 2011), and global incidence has continued to increase over several decades (Melamed and Sorvillo, 2009); septic shock is now the leading cause of death in intensive care units, claiming an estimated 20,000 lives per day worldwide (Daniels, 2011).

Following the withdrawal of Xigris® – the only approved anti-sepsis drug – in 2011, aggressive fluid resuscitation and antibiotic therapy remain the only strictly causal treatments available to septic patients. With the growing threat of widespread antibiotic resistance, the need for new and effective drugs becomes ever more urgent. While research activity has been intensive in recent years, however, translation of pre-clinical results into clinical treatments has been extremely poor. The complexity of the syndrome – compounded by heterogeneous patient populations and the confounding influence of clinical interventions – makes sepsis very difficult to model in pre-clinical systems. Issues in trial design – inappropriate dosing, altered endpoints, lack of patient stratification – have undoubtedly also limited translation of research into the clinic. It is clear that refinement of pre-clinical models, with a strong focus on clinically relevant endpoints, and better communication between research scientists, trial administrators and clinicians will be required to address this tremendous healthcare burden.

Cardiovascular collapse plays a key role in mortality, and correspondingly, pre-clinical and clinical assessment of the impact of interventions on disease progression has been based upon systemic haemodynamics and achieving target blood pressures. In recent years, however, it has become increasingly clear that systemic stabilisation does not necessarily prevent the onset of organ failure, and may actually be achieved at the expense of visceral perfusion (Spronk et al., 2004, Sakr et al., 2004). Sepsis is now increasingly understood to be a ‘disease of the microcirculation’ (Spronk et al., 2004), though pre-clinical models rarely assess this parameter – a further possible explanation for the lack of research translatability. Indeed, any negative impact of interventions on the microcirculation may have been overlooked through excessive focus on the stabilisation of systemic haemodynamics.

In light of growing clinical appreciation of the importance of microvascular perfusion in sepsis, the principal aim of this study was to investigate the changes in microcirculatory function

following the onset of sepsis in a murine model, and to characterise these changes in relation to standard measures of outcome, including haemodynamic function and blood biochemistry. We additionally aimed to use a novel approach to microcirculatory flow assessment, in combination with *in vitro* investigation, to evaluate the roles of two ion channels – TRPV1 and TRPV4 – in sepsis-associated vascular dysfunction.

7.2 Summary of results

The salient findings of this thesis are as follows:

1. Laser speckle contrast imaging can be used in concert with other standard hemodynamic monitoring techniques to robustly assess microcirculatory perfusion in healthy and septic mice.
2. Blood flow in the gut declines significantly in a time-dependent manner, despite systemic stabilisation in both LPS- and CLP-induced murine sepsis.
3. Microcirculatory flow, as quantified in the mesenteric vascular bed, correlates with end organ dysfunction and metabolic disturbance in both sepsis models.
4. Stabilisation of global haemodynamics is likely to occur at the expense of microcirculatory perfusion, and is therefore insufficiently predictive of outcome. By extension, strategies aimed at elevating MAP pharmacologically may adversely affect microcirculatory perfusion.
5. Aggressive fluid resuscitation and topical administration of vasodilatory agents measurably improve microcirculatory perfusion and end organ function, consistent with clinical observations.
6. TRPV1 appears to be protective against cardiovascular dysfunction in sepsis, though this protection is likely to be neuronal in origin, since we found no evidence of TRPV1 expression in vascular cells or macrophages.
7. TRPV4 is expressed in both vascular cells and bone marrow-derived macrophages, and may be sensitised under inflammatory conditions; its blockade, under the conditions of this study, does not markedly affect either haemodynamic or microcirculatory function, either under basal or inflammatory conditions, however.

7.3 Discussion

7.3.1.1 Optimisation of pre-clinical sepsis models

In light of numerous clinical reports of the prognostic value of microcirculatory perfusion (De Backer et al., 2002, Spronk et al., 2004, Sakr et al., 2004, Trzeciak et al., 2007b, Trzeciak et al., 2008, Spanos et al., 2010, Davis et al., 2009), we aimed to establish a robust model for assessing microvascular function in mice. The mesenteric bed was chosen on the strength of several important factors, not least its accessibility and its significant contribution to peripheral vascular resistance. Importantly, low mesenteric flow is known to correlate strongly with multiple organ failure and mortality, both in animal models (Baykal et al., 2000) and in human patients (Takala, 1997). Clinically, gut ischaemia is known as the ‘motor of multiple organ failure’ (Carrico et al., 1986); impaired mesenteric blood flow is associated with intestinal hyperpermeability, and breakdown in barrier function facilitates the leakage of endotoxins and microorganisms into the lymphatic and cardiovascular circulation, which can exacerbate the inflammatory response (Sautner et al., 1998). Furthermore, gastric perfusion is known to correlate well with sublingual perfusion (Marik, 2001) – a robust indicator of outcome in patients (De Backer et al., 2010).

Consistent with original aims, this thesis has demonstrated successful development of a robust and reproducible system for monitoring mesenteric blood flow in healthy and septic mice. Blood flow was found to decline significantly over the course of sepsis in line with the development of metabolic acidosis and organ dysfunction, and in a manner that was sensitive to established clinical interventions. These observations suggest that mesenteric blood flow is a sensitive indicator of syndrome progression, and that incorporation of this endpoint into all pre-clinical studies of intervention efficacy will improve research translatability. Importantly, the impact of interventions shown pre-clinically to improve mesenteric blood flow in sepsis may easily be monitored in the clinic, since both gut tonometry and sidestream dark field imaging are readily available monitoring tools.

7.3.1.2 Mechanisms underlying microvascular flow impairment in sepsis

A dramatic and time-dependent reduction in mesenteric flow was observed after the induction of sepsis by both LPS and CLP. Especially noteworthy was the observation that in both models, hypotension and alterations in cardiac output and heart rate stabilised between 6 h and 24 h after the induction of sepsis, whereas mesenteric blood flow continued to decline significantly over the 24-h time course in both models. This suggests that systemic stabilisation may be preserved at the expense of the microcirculation, as proposed previously (Spronk et al., 2004). The ability of SNP to improve mesenteric blood flow in septic mice gives credence to the

‘shunting theory of sepsis’(Ince and Sinaasappel, 1999), which proposes active shut-down of compliant vascular beds in order to preserve global haemodynamics; while initially an adaptive response to hypovolaemia, prolonged shut-down of the mesenteric bed, in particular, is likely to become mal-adaptive.

This is supported by the observation that in both models of sepsis, mesenteric perfusion, but not haemodynamic performance, was found to decline in line with the development of both acidosis and organ dysfunction. Moreover, administration of noradrenaline – known to raise arterial pressure – substantially diminished mesenteric perfusion in septic mice. These data suggest that prolonged vasopressor use may accelerate organ failure in septic patients; careful monitoring of microcirculatory perfusion is certainly advisable in patients receiving vasopressor therapy, and timing of intervention should be carefully considered. Furthermore, results indicate that treatment strategies targeted at global haemodynamics may have limited efficacy, as demonstrated previously (Gattinoni et al., 1995), and that stabilisation at a marginally hypotensive state may be better tolerated than excessively high target pressures.

While disseminated intravascular coagulation, vessel remodelling, and oedema formation can all influence microvascular function, our results indicate that active peripheral constriction may be a potentially large contributing factor in the observed ‘oxygen extraction deficit’. This would certainly account for the measurements of normal mixed venous SO_2 levels despite severe local tissue dysoxia, as observed both in this study and in septic patients (Ince and Sinaasappel, 1999), as well as the significant increase in blood flow elicited by SNP. While we did not observe substantial organ oedema in septic mice, or any beneficial impact of heparin therapy, the contribution of coagulation and plasma extravasation to the microcirculatory flow impairment recorded remains unknown; it is possible that more sensitive monitoring techniques or alternative anti-coagulant therapy would have uncovered a role for these processes. Irrespective of the mechanisms involved, however, the observations recorded in this thesis highlight the importance of measuring loco-regional perfusion directly, rather than using global surrogates. Numerous studies have demonstrated that restoration of global oxygen delivery does not necessarily enhance regional perfusion (Gattinoni et al., 1995, LeDoux et al., 2000, Bourgoin et al., 2005, Jhanji et al., 2009, Dubin et al., 2009), and we conclude that microvascular blood flow is a more sensitive and clinically relevant indicator of syndrome progression than haemodynamic indices alone.

7.3.1.3 A technological advance

This thesis has described a novel approach to microvascular blood flow assessment *in situ*, that can be incorporated into a multi-parameter monitoring system, facilitating comprehensive evaluation of all levels of the cardiovascular system in a single mouse. Blood flow and core temperature were found to remain stable throughout the blood flow recording period, validating the use of this model for assessment of vasoactive responses to acute pharmacological intervention, and absolute values in both naïve and septic states were highly reproducible. The novel application of laser speckle contrast imaging technology described in this study enables real-time, *in situ* visualisation of blood flow across the entire mesenteric bed, with no dissection of perivascular tissue and minimal mechanical disruption to the vessels. This represents a significant advantage over traditional laser Doppler techniques that are usually limited to measurements in single vessels, often requiring extensive microdissection and probe-tissue contact, with significantly lower processing speeds. Additionally, this technique does not require the use of fluorescent dyes necessary for intravital microscopy. Furthermore, it allows quantification of flow within specified regions of interest, both at baseline and following topical or intravenous administration of a pharmacological agent. As such, the model represents a practical substitute to the sidestream dark field imaging technique that is used clinically, while circumventing the need for high-level operating expertise, as well as issues of inter-operator variability, user bias and probe-tissue contact artefacts. Furthermore, this microcirculatory blood flow model is not limited to sepsis research, but has wider applications for *in situ* investigation of thrombosis or acute vasoactivity; it can also be adapted to larger animals, in which the administration of greater fluid volumes facilitates hyperdynamic modelling.

7.3.1.4 Evaluation of TRPV ion channels as potential regulators of cardiovascular function in sepsis

7.3.1.4.1 TRPV1

Based on numerous previous studies describing a protective role for TRPV1 in sepsis (Clark et al., 2007, Wang et al., 2008a, Fernandes et al., 2012, Wang and Wang, 2013), we aimed to determine the impact of TRPV1 deletion and activation on microcirculatory blood flow and blood biochemistry in the LPS model of sepsis.

Our data demonstrate that TRPV1 gene ablation is associated with exaggerated microvascular impairment (and correspondingly with evidence of exaggerated organ dysfunction and metabolic acidosis), consistent with previous reports. Whether endogenous activation of

TRPV1 in sepsis contributes to local vasodilatation and the maintenance of regional perfusion is unclear, and warrants further investigation.

Given that TRPV1 has reported to be expressed in endothelial cells, and to contribute directly to endothelium-dependent vasodilatation (Kark et al., 2008, Yang et al., 2010a), we decided to investigate whether TRPV1 may mediate vasculo-protective effects in sepsis via its expression in the vascular endothelium. We hypothesised that inflammation-induced upregulation/sensitisation of TRPV1 may contribute to the maintenance of regional perfusion. Despite finding robust evidence of TRPV1 mRNA in aortic lysates and endothelial cells, however, we have found no evidence of functional TRPV1 protein expression in either isolated endothelial or smooth muscle cells, or in bone marrow-derived macrophages, in either basal or inflammatory conditions. While these data do not preclude the possibility of induced endothelial TRPV1 expression under certain pathological conditions not tested in this study, or indeed of functional expression in specific subpopulations of endothelial cells (from the microvasculature, for example), our results suggest that *in vivo* observations of a protective role for TRPV1 in sepsis-associated vascular dysfunction are not due to direct activity in the vasculature itself.

Importantly, a thorough investigation of widely used anti-TRPV1 antibodies revealed limited selectivity for the receptor. These data call into question the validity of numerous reports of TRPV1 expression that have not been validated using appropriate controls.

7.3.1.4.2 TRPV4

During our *in vitro* investigation of endothelial TRPV1 expression, we observed strong evidence of TRPV4 activity in a range of different endothelial cell lines. Based on observations of LPS-induced sensitisation of endothelial TRPV4 activity, and in light of previous studies linking excessive TRPV4 activation with profound hypotension, endothelial failure, circulatory collapse (Willette et al., 2008) and hyperpermeability (Yin et al., 2008, Alvarez et al., 2006, Wu et al., 2009, Hamanaka et al., 2010), we hypothesised that TRPV4 may contribute to haemodynamic and vascular dysfunction during sepsis. Specifically, we expected blockade of TRPV4 activity to attenuate the circulatory dysfunction that underlies sepsis pathogenesis.

We observed no dramatic effects on either haemodynamic or microcirculatory function in either antagonist-treated or TRPV4 KO mice. Surprisingly, given that TRPV4 has been implicated in both NO- and EDHF-mediated vasodilatory responses to physical and chemical stimuli in both conduit and resistance arteries (Kohler et al., 2006, Hartmannsgruber et al., 2007, Loot et al., 2008, Zhang et al., 2009, Mendoza et al., 2010, Adapala et al., 2011, Rath et al., 2012, Sonkusare et al., 2012, Ma et al., 2013a), TRPV4 KO mice exhibited trends towards

basal hypotension, and, like antagonist-treated mice, exhibited a trend towards exaggerated LPS-induced hypotension. A possible explanation is that loss of TRPV4 leads to compensatory upregulation of alternative vasodilatory pathways during development. Previous reports have shown that blockade of the TRPV4-CYP450 epoxygenase axis only inhibits flow-induced vasodilatation in the presence of eNOS and prostacyclin antagonists (Loot et al., 2008), suggesting that blockade of one endothelium-dependent vasodilatory pathway can lead to acute upregulation of a compensatory pathway. Alternatively, since plasma osmolality is known to increase in sepsis (Jochberger et al., 2009), and hypertonicity inhibits TRPV4 activity (Strotmann et al., 2000), it is possible that under septic conditions, TRPV4 activity is inhibited such that gene deletion or pharmacological antagonism will produce no further effect.

Importantly, while both antagonist-treated and TRPV4 KO mice exhibited exaggerated LPS-induced hypotension relative to vehicle-treated controls, mesenteric perfusion was not worse in these mice; in fact, antagonist-treated mice exhibited trends towards improved microcirculatory blood flow after the induction of sepsis, and the development of metabolic acidosis appeared less severe in mice lacking TRPV4. These observations emphasise the low prognostic value of haemodynamic indices, which may be preserved at the expense of regional perfusion, and partially support the hypothesis that loss of TRPV4 signalling attenuates sepsis pathophysiology.

7.4 Study limitations and future directions

7.4.1 Murine models of sepsis

Because murine models lend themselves well to genetic modification, they currently represent the only viable option for conducting mechanistic pre-clinical proof-of-concept research. Nonetheless, recent reports of divergent immune cell genomic responses to inflammation between mice and men (Seok et al., 2013) have called into question the validity of mouse models of inflammatory conditions, including sepsis. While it is clear that rodents do not necessarily recapitulate all the genomic responses to sepsis encountered in humans, they do exhibit highly similar pathophysiological sequelae (Osuchowski et al., 2014), most notably cardiovascular dysfunction, which is known to be a key feature of sepsis pathogenesis in humans (Parrillo, 1990). As such, the mouse represents a highly useful tool for early mechanistic studies in a mammalian system that largely mirrors the critical sepsis-induced pathophysiological changes that occur in human patients.

Given the obvious limitations, it is essential that we optimise the systems available to us by limiting confounding influences and employing animal models to their full capacity, extracting as much information as possible from a single individual. With this in mind, we aimed to

establish a robust model for assessing microcirculatory perfusion in a septic mouse, and to incorporate this model into a multi-parameter monitoring system for direct comparison (in the same animal) of microvascular and global haemodynamic parameters. To our knowledge, this study represents the first comprehensive multi-parameter investigation of sepsis-associated cardiovascular derangements in a single animal. Results demonstrate that a number of biological measurements can be performed in a single mouse without compromising data quality or reproducibility, and without incurring undue suffering, which can lead to stress-induced artefacts. While results, in sepsis research in particular, should always be interpreted with caution, and never over-extrapolated, proof-of-concept studies in well-designed mouse models with clinically relevant endpoints can provide valuable information on the likely impact of a particular intervention on a physiological system, and can inform the design of relevant clinical trials. This is particularly important in the later stages of sepsis, where mortality rates are unacceptably high, and where there is a strong requirement for adjunct therapies.

A further limitation of the mouse model is its size, which precludes the administration of large volumes of fluid: it is consequently difficult to compare well resuscitated human patients with murine models of sepsis. The observed improvement in perfusion and organ function in response to fluid resuscitation, however, suggests that the model used here is clinically relevant and may be adapted to a comparatively hyperdynamic model of sepsis (Hollenberg et al., 2001). The extent to which a mouse can reflect the truly hyperdynamic state that occurs in continuously infused patients is undoubtedly limited. Murine models may be used effectively to understand the pathophysiological changes that occur when sepsis is allowed to progress unimpeded, however, and to establish the impact of pharmacological or genetic modification in the absence of other interventions. To mimic more closely the resuscitated clinical situation, larger species should be used, where administration of large volumes of fluid in addition to pharmacological agents is more feasible. Nonetheless, the data obtained emphasise the importance of using microcirculatory blood flow to assess a patient's response to interventional treatment; use of this endpoint in pre-clinical models will help to determine more accurately the impact of a pharmacological intervention on overall health.

7.4.2 TRPV4 KO mice

Owing to breeding problems we unfortunately did not obtain sufficient numbers of TRPV4 KO mice to conduct a sufficiently well-powered study. An additional cohort of mice were included from the parent colony at the Wolfson Centre, King's College London, though these were neither littermates nor age-matched, limiting the strength of comparison. It is unclear why these breeding issues arose in our animal unit, though sufficient numbers may be generated

for future work through homozygous crosses, as long as offspring are back-crossed every few generations to prevent genetic drift.

7.4.3 TRPV4 antagonism

Because the TRPV4 antagonist HC-067047 has not been well characterised *in vivo*, and has only been used for acute studies (Everaerts et al., 2010, Materazzi et al., 2012), its pharmacokinetic properties over the 24 h following a single i.p. injection are unknown. It is possible that the dose used in this study was insufficient to achieve complete and sustained TRPV4 blockade throughout the duration of the study, and that changes would have been detected at higher concentrations or with repeated dosing. Both options confer significant limitations: higher concentrations of HC-067047 are known to induce adverse effects, including piloerection and hunching (Everaerts et al., 2010) and the high percentage of DMSO that would be required to dissolve the compound may itself cause cytotoxicity; secondly, repeated handling and injection would certainly preclude meaningful haemodynamic recording, although this approach may be useful for further microcirculatory and biochemical studies. Alternatively, administration of orally available TRPV4 antagonists (Thorneloe et al., 2012) via supplementation of drinking water or a subcutaneous pump may represent a more attractive option, and could be used to elucidate further the role of TRPV4 in sepsis-associated vascular dysfunction.

7.4.4 Molecular investigation

Further biochemical investigation of TRPV1 and TRPV4 expression would be particularly informative. Identification of a more selective TRPV1 antibody could help to elucidate the exact localisation of the receptor; immunohistochemical analysis of mesenteric sections, for example, would be useful in determining whether TRPV1 is in fact expressed in mesenteric smooth muscle and/or endothelium, and whether its expression changes in these tissues and in perivascular nerves after the induction of sepsis, or whether it is simply channel activity that is altered.

Investigation of the tissue localisation of TRPV4, and expression levels before and after the induction of sepsis, would be similarly informative, and would clarify whether LPS-induced changes in activity are due to sensitisation or upregulation. Further investigation of TRPV4 expression and activity in macrophages, using fluorescence-associated cell sorting, would also shed light on the immunological function of this channel.

Furthermore, it would be interesting to evaluate the extent of any vascular or cardiac remodelling in antagonist-treated or KO mice.

7.4.5 Further applications of the microvascular blood flow model

Having established the prognostic sensitivity and clinical relevance of microvascular blood flow in sepsis, it would be interesting to use the model for assessment of alternative potential interventions. Since neither TRPV1 nor TRPV4 appear to be particularly attractive drug targets, owing to widespread expression and function, and the potential acute upregulation of compensatory pathways, assessment of more fruitful targets or interventions using this system could yield exciting results. Furthermore, simultaneous blood pressure recording (either through invasive catheterisation or through incorporation of telemetric receivers into the blood flow set-up) would help to elucidate further the relationship between alterations in global haemodynamics and regional perfusion. It would be interesting to measure blood flow in more critical microvascular beds, using transcranial Doppler probes, for example, or markers of cardiac perfusion, such as cardiac troponin, for further evaluation of the relative prognostic sensitivity of microvascular perfusion versus global haemodynamics.

Use of alternative anti-coagulant drugs may also provide further mechanistic insight into the relative contributions of microthrombus formation and active vasoconstriction in sepsis-associated microcirculatory impairment. It would also be interesting to apply the mesenteric model to *in situ* assessments of vasoactivity (since myographical methods are limited by the requirement for significant microdissection) and thrombus formation/resolution in more general studies.

An additional limitation of this investigation is the restrictive 24-h time course, which prevents longer-term investigation of the impact of interventions on sepsis pathogenesis. While extension of the study period is legally prohibited, a more comprehensive assessment of time-dependent physiological changes within the 24-h window may be possible. Further validation of the ear blood flow model, or use of a non-invasive rectal Doppler probe, may facilitate continuous or repeated-measures evaluation of changes in microvascular blood flow over time.

7.5 Concluding remarks

In summary, this thesis has demonstrated that microcirculatory perfusion correlates more closely with end organ damage and metabolic dysfunction than global haemodynamics, and that the latter should not be used as an indicator of outcome in isolation. Quantification of microcirculatory blood flow, as described in this thesis, provides a humane endpoint, whilst simultaneously improving the clinical translatability of pre-clinical sepsis studies.

While *in vitro* data provided compelling evidence of a vasoregulatory role for TRPV4 in sepsis, our *in vivo* data did not support this hypothesis. Whether upregulation of alternative pathways can compensate for its loss, or whether TRPV4 is tonically inhibited during sepsis such that further inhibition is impossible, is not clear. Regardless, the channel does not appear to represent a viable target in the treatment of sepsis-associated vascular dysfunction.

While TRPV1 did appear to play a protective role in the development of sepsis, its ubiquitous expression, not least in sensory nerves, limit the possibility of using channel activation as a potential therapeutic strategy. Further mechanistic investigation of downstream mediators of TRPV1 activation may be more fruitful.

Overall, data obtained in this study have advanced understanding of the pathophysiological changes elicited in sepsis: we have demonstrated that mesenteric perfusion is substantially reduced after the onset of sepsis, in line with the development of organ dysfunction and metabolic acidosis, despite (or perhaps because of) systemic stabilisation. Using a novel approach to microcirculatory flow monitoring, we have demonstrated the prognostic value of mesenteric blood flow as an endpoint in sepsis, and suggest that pre-clinical models that incorporate this parameter will facilitate more clinically relevant studies.

CHAPTER 8 | REFERENCES

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